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(54) Title: RECOMBINASE–ACTIVATABLE AAV PACKAGING CASSETTES FOR USE IN THE PRODUCTION OF AAV VECTORS

(57) Abstract

The invention provides recombinase–activatable adeno–associated virus (AAV) packaging cassettes, and packaging cell lines comprising such cassettes, which are useful for generating recombinant AAV particles that can effectively mediate delivery of genes of interest to mammalian cells.

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RECOMBINASE-ACTIVATABLE AAV PACKAGING CASSETTES FOR USE IN THE PRODUCTION OF AAV VECTORS

Technical Field of the Invention

This invention relates to materials and methods used for the generation of viral vectors, particularly recombinant adeno-associated virus (AAV) vectors. More specifically, the invention relates to recombinase-activatable AAV packaging cassettes and cell lines comprising such cassettes for use in the production of AAV vectors.

Background

Vectors based on adeno-associated virus (AAV) are believed to have utility for gene therapy but a significant obstacle has been the difficulty in generating such vectors in amounts that would be clinically useful for human gene therapy applications. This is a particular problem for *in vivo* applications such as direct delivery to the lung. Another important goal in the gene therapy context, discussed in more detail herein, is the production of vector preparations that are essentially free of replication-competent virions. The following description briefly summarizes studies involving adeno-associated virus and AAV vectors, and then describes a number of novel improvements according to the present invention that are useful for efficiently generating high titer recombinant AAV vector (rAAV) preparations suitable for use in gene therapy.

Adeno-associated virus is a defective parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. General reviews of AAV may be found in, for example, Carter, 1989, Handbook of Parvoviruses, Vol. I, pp. 169-228, and Berns, 1990, Virology, pp. 1743-1764, Raven Press, (New York). Examples of co-infecting viruses that provide helper functions for AAV growth and replication are adenoviruses, herpesviruses and, in some cases, poxviruses such as vaccinia. The nature of the helper function is not entirely known but it appears that the helper virus indirectly renders the cell permissive for AAV replication. This belief is supported by the observation that AAV replication may occur at low efficiency in the absence of

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helper virus co-infection if the cells are treated with agents that are either genotoxic or that disrupt the cell cycle.

Although AAV may replicate to a limited extent in the absence of helper virus in these unusual conditions more generally infection of cells with AAV in the absence of helper functions results in the proviral AAV genome integrating into the host cell genome. If these cells are superinfected with a helper virus such as adenovirus, the integrated AAV genome can be rescued and replicated to yield a burst of infectious progeny AAV particles. The fact that integration of AAV appears to be efficient suggests that AAV would be a useful vector for introducing genes into cells for uses such as human gene therapy.

AAV has a very broad host range without any obvious species or tissue specificity and can replicate in virtually any cell line of human, simian or rodent origin provided that an appropriate helper is present. AAV is also relatively ubiquitous and has been isolated from a wide variety of animal species including most mammalian and several avian species.

AAV is not associated with the cause of any disease. Nor is AAV a transforming or oncogenic virus, and integration of AAV into the genetic material of human cells generally does not cause significant alteration of the growth properties or morphological characteristics of the host cells. These properties of AAV also recommend it as a potentially useful human gene therapy vector because most of the other viral systems proposed for this application, such as retroviruses, adenoviruses, herpesviruses, or poxviruses, are disease-causing.

AAV particles are comprised of a proteinaceous capsid having three capsid proteins, VP1, VP2 and VP3, which enclose a DNA genome. The AAV DNA genome is a linear single-stranded DNA molecule having a molecular weight of about 1.5 x 10^6 daltons and a length of approximately 4680 nucleotides. Individual particles package only one DNA molecule strand, but this may be either the "plus" or "minus" strand. Particles containing either strand are infectious and replication occurs by conversion of the parental infecting single strand to a duplex form and subsequent amplification of a large pool of duplex molecules from which progeny single strands are displaced and packaged into capsids. Duplex or single-strand copies of AAV genomes can be inserted into bacterial plasmids or phagemids and transfected into adenovirus-infected cells; these techniques have facilitated the study of AAV genetics and the development of AAV vectors.

The AAV genome, which encodes proteins mediating replication and encapsidation of the viral DNA, is generally flanked by two copies of inverted terminal repeats (ITRs). In the case of AAV2, for example, the ITRs are each 145 nucleotides in length, flanking a unique sequence region of about 4470 nucleotides that contains two main open reading frames for the *rep* and *cap* genes (Srivastava et al., 1983, J. Virol., 45:555-564; Hermonat et al., J. Virol. 51:329-339;

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Tratschin et al., 1984a, J. Virol., 51:611-619). The AAV2 unique region contains three transcription promoters p5, p19, and p40 (Laughlin et al., 1979, Proc. Natl. Acad. Sci. USA, 76:5567-5571) that are used to express the *rep* and *cap* genes. The ITR sequences are required *in cis* and are sufficient to provide a functional origin of replication (*ori*), signals required for integration into the cell genome, and efficient excision and rescue from host cell chromosomes or recombinant plasmids. It has also been shown that the ITR can function directly as a transcription promoter in an AAV vector (Flotte et al., 1993, *supra*).

The *rep* and *cap* gene products are required *in trans* to provide functions for replication and encapsidation of viral genome, respectively. The *rep* gene is expressed from two promoters, p5 and p19, and produces four proteins. Transcription from p5 yields an unspliced 4.2 kb mRNA encoding a first Rep protein (Rep78), and a spliced 3.9 kb mRNA encoding a second Rep protein (Rep68). Transcription from p19 yields an unspliced mRNA encoding a third Rep protein (Rep52), and a spliced 3.3 kb mRNA encoding a fourth Rep protein (Rep40). Thus, the four Rep proteins all comprise a common internal region sequence but differ in their amino and carboxyl terminal regions. Only the large Rep proteins (i.e. Rep78 and Rep68) are required for AAV duplex DNA replication, but the small Rep proteins (i.e. Rep52 and Rep40) appear to be needed for progeny, single-strand DNA accumulation (Chejanovsky & Carter, 1989, Virology 173:120-128). Rep68 and Rep78 bind specifically to the hairpin conformation of the AAV ITR and possess several enzyme activities required for resolving replication at the AAV termini. Rep52 and Rep40 have none of these properties. Recent reports by C. Hölscher et al. (1994, J. Virol. 68:7169-7177; and 1995, J. Virol. 69:6880-6885) suggest that expression of Rep78 or Rep 68 may in some circumstances be sufficient for infectious particle formation.

The Rep proteins, primarily Rep78 and Rep68, also exhibit pleiotropic regulatory activities including positive and negative regulation of AAV genes and expression from some heterologous promoters, as well as inhibitory effects on cell growth (Tratschin et al., 1986, Mol. Cell. Biol. 6:2884-2894; Labow et al., 1987, Mol. Cell. Biol., 7:1320-1325; Khleif et al., 1991, Virology 181:738-741). The AAV p5 promoter is negatively auto-regulated by Rep78 or Rep68 (Tratschin et al., 1986, Mol. Cell. Biol. 6:2884-2894). Due to the inhibitory effects of expression of *rep* on cell growth, constitutive expression of *rep* in cell lines has not been readily achieved. For example, Mendelson et al. (1988, Virology, 166:154-165) reported very low expression of some Rep proteins in certain cell lines after stable integration of AAV genomes.

The capsid proteins VP1, VP2, and VP3 share a common overlapping sequence, but VP1 and VP2 contain additional amino terminal sequences. All three proteins are encoded by the same *cap* gene reading frame typically expressed from a spliced 2.3 kb mRNA transcribed from the p40

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promoter. VP2 and VP3 can be generated from this mRNA by use of alternate initiation codons. Generally, transcription from p40 yields a 2.6 kb precursor mRNA which can be spliced at alternative sites to yield two different transcripts of about 2.3 kb. VP2 and VP3 can be encoded by either transcript (using either of the two initiation sites), whereas VP1 is encoded by only one of the transcripts. VP3 is the major capsid protein, typically accounting for about 90% of total virion protein. VP1 is coded from a minor mRNA using a 3' donor site that is 30 nucleotides upstream from the 3' donor used for the major mRNA that encodes VP2 and VP3. All three proteins are required for effective capsid production. Mutations which eliminate all three proteins (Cap-negative) prevent accumulation of single-strand progeny AAV DNA, whereas mutations in the VP1 amino-terminus ("Lip-negative" or "Inf-negative") can permit assembly of single-stranded DNA into particles but the infectious titer is greatly reduced.

The genetic analysis of AAV that was highlighted above was largely based upon mutational analysis of AAV genomes cloned into bacterial plasmids. In early work, molecular clones of infectious genomes of AAV were constructed by insertion of double-strand molecules of AAV into plasmids by procedures such as GC tailing (Samulski et al., 1982, Proc. Natl. Acad. Sci. USA, 79:2077-2081), addition of synthetic linkers containing restriction endonuclease cleavage sites (Laughlin et al., 1983, Gene, 23:65-73) or by direct, blunt-end ligation (Senpathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). Transfection of such AAV recombinant plasmids into mammalian cells that were also infected with an appropriate helper virus, such as adenovirus, resulted in rescue and excision of the AAV genome free of any plasmid sequence, replication of the rescued genome and generation of progeny infectious AAV particles. This provided the basis for performing genetic analysis of AAV as summarized above and permitted construction of AAV transducing vectors.

Based on the genetic analysis, the general principles of AAV vector construction were defined as reviewed recently (Carter, 1992, Current Opinions in Biotechnology, 3:533-539; Muzyczka, 1992, Curr. Topics in Microbiol. and Immunol., 158:97-129). AAV vectors are generally constructed in AAV recombinant plasmids by substituting portions of the AAV coding sequence with foreign DNA to generate a recombinant AAV (rAAV) vector or "pro-vector". In the vector, the terminal (ITR) portions of the AAV sequence must generally be retained intact because these regions are generally required *in cis* for several functions, including excision from the plasmid after transfection, replication of the vector genome and integration and rescue from a host cell genome. In some situations, providing a single ITR may be sufficient to carry out the functions normally associated with two wild-type ITRs (see, e.g., Samulski, R.J. et al., WO 94/13788, published 23 June 1994). The vector can then be packaged into an AAV particle to

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generate an AAV transducing virus by transfection of the vector into cells that are infected by an appropriate helper virus such as adenovirus or herpesvirus; provided that, in order to achieve replication and encapsidation of the vector genome into AAV particles, the vector must be complemented for any AAV functions required *in trans*, particularly *rep* and *cap*, that were deleted in construction of the vector.

Such AAV vectors are among a small number of recombinant virus vector systems which have been shown to have utility as *in vivo* gene transfer agents (reviewed in Carter, 1992, Current Opinion in Biotechnology, 3:533-539; Muzcyzka, 1992, Curr. Top. Microbiol. Immunol. 158:97-129) and thus are potentially of great importance for human gene therapy. AAV vectors are capable of high-frequency transduction and expression in a variety of cells including cystic fibrosis (CF) bronchial and nasal epithelial cells (see, e.g., Flotte et al., 1992a, Am. J. Respir. Cell Mol. Biol. 7:349-356; Egan et al., 1992, Nature, 358:581-584; Flotte et al., 1993a, J. Biol. Chem. 268:3781-3790; and Flotte et al., 1993b, Proc. Natl. Acad. Sci. USA, 93:10163-10167); human bone marrow-derived erythroleukemia cells (see, e.g., Walsh et al., 1992, Proc. Natl. Acad. Sci. USA, 89:7257-7261); as well as brain, eye and muscle cells. AAV may not require active cell division for transduction and expression which would be another clear advantage over retroviruses, especially in tissues such as the human airway epithelium where most cells are terminally differentiated and non-dividing.

There are at least two desirable features of any AAV vector designed for use in human gene therapy. First, the transducing vector must be generated at titers sufficiently high to be practicable as a delivery system. This is especially important for gene therapy stratagems aimed at *in vivo* delivery of the vector. For example, it is likely that for many desirable applications of AAV vectors, such as treatment of cystic fibrosis by direct *in vivo* delivery to the airway, the required dose of transducing vector may be in excess of 10^{10} particles. Secondly, the vector preparations are preferably essentially free of wild-type AAV virus (or any replication-competent AAV). The attainment of high titers of AAV vectors has been difficult for several reasons including preferential encapsidation of wild-type AAV genomes (if they are present or generated by recombination), and the difficulty in generating sufficient complementing functions such as those provided by the wild-type *rep* and *cap* genes. Useful cell lines expressing such complementing functions have been especially difficult to generate, in part because of pleiotropic inhibitory functions associated with the *rep* gene products. Thus, cell lines in which the *rep* gene is integrated and expressed tend to grow slowly or express *rep* at very low levels.

The first AAV vectors described contained foreign reporter genes such as *neo*, *cat* or *dhfr* expressed from AAV transcription promoters or an SV40 promoter (Tratschin et al., 1984b, Mol.

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Cell. Biol. 4:2072-2081; Hermonat & Muzyczka, 1984, Proc. Natl. Acad. Sci. USA, 81:6466-6470; Tratschin et al., 1985, Mol. Cell. Biol. 5:3251-3260; McLaughlin et al., 1988, J. Virol., 62:1963-1973; Lebkowski et al., 1988 Mol. Cell. Biol., 7:349-356). These vectors were packaged into AAV-transducing particles by co-transfection into adenovirus-infected cells together with a second "packaging plasmid" containing the AAV *rep* and *cap* genes expressed from the wild-type AAV transcription promoters. Several strategies have been employed in attempts to prevent encapsidation of the packaging plasmid. In some cases, (Hermonat & Muzyczka, 1984; McLaughlin et al., 1988) a large region of bacteriophage lambda DNA was inserted into the packaging plasmid within the AAV sequence to generate an oversized genome that could not be packaged. In other cases, (Tratschin et al., 1984b; Tratschin et al., 1985, Lebkowski et al., 1988), the packaging plasmid had deleted the ITR regions of AAV so that it could not be excised and replicated and thus could not be packaged. All of these approaches failed to prevent generation of particles containing replication-competent AAV DNA and also failed to generate effective high titers of AAV transducing particles. Indeed, titers of not more than 10^4 infectious particles per ml were cited by Hermonat & Muzyczka, 1984.

In many studies, the presence of overlapping homology between AAV sequences present in the vector and packaging plasmids resulted in the production of replication-competent AAV particles. It was shown by Senapathy and Carter (1984, J. Biol. Chem. 259:4661-4666) that the degree of recombination in such a system is approximately equivalent to the degree of sequence overlap. It was suggested in a review of the early work (Carter 1989, Handbook of Parvoviruses, Vol. II, pp. 247-284, CRC Press, Boca Raton, FL) that titers of 10^6 infectious particles per ml might be obtained, but this was based on the above-cited studies in which large amounts of replication-competent AAV contaminated the vector preparation. Such vector preparations containing replication-competent AAV will generally not be preferred for human gene therapy. Furthermore, these early vectors exhibited low transduction efficiencies and did not transduce more than 1 or 2% of cells in cultures of various human cell lines even though the vectors were supplied at multiplicities of up to 50,000 particles per cell. This may have reflected in part the contamination with replication-competent AAV particles and the presence of the AAV *rep* gene in the vector. Furthermore, Samulski et al. (1989, J. Virol. 63:3822-3828) showed that the presence of wild-type AAV significantly enhanced the yield of packaged vector. Thus, in packaging systems where the production of wild-type AAV is eliminated, the yield of packaged vector may actually be decreased. Nevertheless, for use in any human clinical application it will be preferable to essentially eliminate production of replication-competent AAV.

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Additional studies (McLaughlin et al., 1988; Lebkowski et al., 1988) attempting to generate AAV vectors lacking the AAV *rep* or *cap* genes still generated replication-competent AAV and still produced very low transduction frequencies on human cell lines. Thus, McLaughlin et al., 1988 reported that AAV *rep*-negative *cap*-negative vectors containing the *neo* gene packaged with the same packaging plasmid used earlier by Hermonat & Muzyczka (1984) still contained replication-competent AAV. As a consequence, it was only possible to use this virus at a multiplicity of 0.03 particles per cell (i.e., 300 infectious units per 10,000 cell) to avoid double hits with vector and wild-type particles. Thus, when 32,000 cells were infected with 1000 infectious units, an average of 800 geneticin-resistant colonies was obtained. Although this was interpreted as demonstrating that the virus was capable of yielding a transduction frequency of 80%, in fact only 2.5% of the cells were transduced. Thus the effectively useful titer of this vector was limited. Furthermore, this study did not demonstrate that the actual titer of the vector preparation was any higher than those obtained previously by Hermonat & Muzyczka (1984). Similarly, Lebkowski et al., 1988, packaged AAV vectors which did not contain either a *rep* or *cap* gene, using an *ori*-negative packaging plasmid (pBa1A) identical to that used earlier by Tratschin et al., (1984b, 1985), and reported transduction frequencies that were similarly low, in that for several human cell lines not more than 1% of the cells could be transduced to geneticin resistance even with their most concentrated vector stocks. Lebkowski et al., (1988) did not report the actual vector titers in a meaningful way but the biological assays, showing not more than 1% transduction frequency when 5×10^6 cells were exposed to three ml of vector preparation, indicate that the titer was less than 2×10^4 geneticin resistant units per ml. Also, the pBa1A packaging plasmid contains overlapping homology with the ITR sequence in the vector and can lead to generation of replication-competent AAV by homologous recombination.

Laface et al. (1988) used the same vector as that used by Hermonat & Muzyczka (1984) prepared in the same way and obtained a transduction frequency of 1.5% in murine bone marrow cultures, again showing very low titer.

Samulski et al. (1987, J. Virol., 61:3096-3101) constructed a plasmid called pSub201 which contained an intact AAV genome in a bacterial plasmid but which had a deletion of 13 nucleotides at the extremity of each ITR and thus was rescued and replicated less efficiently than other AAV plasmids that contained the entire AAV genome. Samulski et al. (1989, J. Virol., 63:3822-3828) constructed AAV vectors based on pSub201 but deleted for *rep* and *cap* and containing either a *hyg* or *neo* gene expressed from an SV40 early gene promoter. They packaged these vectors by co-transfection with a packaging plasmid called pAAV/Ad which consisted of the entire AAV nucleotide sequence from nucleotide 190 to 4490 enclosed at either end with one copy

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of the adenovirus ITR. In this packaging plasmid the AAV *rep* and *cap* genes were expressed from their native AAV promoters (i.e. p5, p19 and p40, as discussed above). The function of the adenovirus ITR in pAAV/Ad was thought to enhance the expression level of AAV capsid proteins. However, *rep* is expressed from its homologous promoter and is negatively regulated and thus its expression is limited. Using their encapsidation system, Samulski et al. generated AAV vector stocks that were substantially free of replication-competent AAV but had transducing titers of only 3×10^4 hygromycin-resistant units per ml of supernatant. When a wild-type AAV genome was used in the packaging plasmid, the titer of the AAV vector prep was increased to 5×10^4 hygromycin-resistant units per ml. The low titer produced in this system thus appears to have been due in part to the defect in the ITR sequences of the basic pSub201 plasmid used for vector construction and in part due to limiting expression of AAV genes from pAAV/Ad. In an attempt to increase the titer of the AAVneo vector preparation, Samulski et al. generated vector stocks by transfecting, in bulk, thirty 10-cm dishes of 293 cells and concentrating the vector stock by banding in CsCl. This produced an AAVneo vector stock containing a total of 10^8 particles as measured by a DNA dot-blot hybridization assay. When this vector stock was used at multiplicities of up to 1,000 particles per cell, a transduction frequency of 70% was obtained. This suggests that the particle-to-transducing ratio is about 500 to 1,000 particles since at the ratio of one transducing unit per cell the expected proportion of cells that should be transduced is 63% according to the Poisson distribution.

Although the system of Samulski et al. (1989), using the vector plasmid pSub201 and the packaging plasmid pAAV/Ad, did not have overlapping AAV sequence homology between the two plasmids, there is overlapping homology at the XbaI sites and recombination of these sites can lead to the generation of complete replication-competent AAV. That is, although overlapping homology of AAV sequence is not present, the complete AAV sequence is contained within the two plasmids and the plasmids share a short (non-AAV) sequence that might facilitate recombination to generate replication-competent AAV, which is undesirable. That this class of recombination occurs in AAV plasmids was shown by Senapathy & Carter (1984, J. Biol. Chem. 259:4661-4666). Given the problems of low titer, and the capability of generating wild-type recombinants, the system described by Samulski et al., 1989, does not have practical utility for human gene therapy.

Several other reports have described AAV vectors. For example, Srivastava et al., (1989, Proc. Natl. Acad. Sci. USA, 86:8078-8082) described an AAV vector based on the pSub201 plasmid of Samulski et al. (1987), in which the coding sequences of AAV were replaced with the coding sequences of another parvovirus, B19. This vector was packaged into AAV particles using

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the pAAV/Ad packaging plasmid to generate a functional vector, but titers were not reported. This system was based on pSub201 and thus suffers from the defect described above for this plasmid. Second, the vector and the packaging plasmid contained overlapping AAV sequences (the ITR regions) and thus recombination yielding contaminating wild-type virus is highly likely.

Chatterjee et al. (1991, Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 85-89), Wong et al. (1991, Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 183-189), and Chatterjee et al. (1992, Science, 258:1485-1488) describe AAV vectors designed to express antisense RNA directed against infectious viruses such as HIV or Herpes simplex virus. However, these authors did not report any titers of their AAV vector stocks. Furthermore, they packaged their vectors using an *ori*-negative packaging plasmid analogous to that used by Tratschin et al. (1984b, 1985) containing the Bal A fragment of the AAV genome and therefore their packaging plasmid contained AAV vector sequences that have homology with AAV sequences that were present in their vector constructs. This will also lead to generation of replication-competent AAV. Thus, Chatterjee et al., and Wong et al., used a packaging system known to give only low titer and which can lead to generation of replication-competent AAV genomes because of the overlapping homology in the vector and packaging sequences.

Other reports have described the use of AAV vectors to express genes in human lymphocytes (Muro-Cacho et al., 1992, J. Immunotherapy, 11:231-237) or a human erythroid leukemia cell line (Walsh et al., 1992, Proc. Natl. Acad. Sci. USA, 89:7257-7261) with vectors based on the pSub201 vector plasmid and pAAV/Ad packaging plasmid. Again, titers of vector stocks were not reported and were apparently low because a selective marker gene was used to identify those cells that had been successfully transduced with the vector.

Transduction of human airway epithelial cells, grown *in vitro* from a cystic fibrosis patient, with an AAV vector expressing the selective marker gene *neo* from the AAV p5 promoter was reported (Flotte et al., 1992, Am. J. Respir. Cell. Mol. Biol. 7:349-356). In this study the AAV*neo* vector was packaged into AAV particles using the pAAV/Ad packaging plasmid. Up to 70% of the cells in the culture could be transduced to geneticin resistance and the particle-to-transducing ratio was similar to that reported by Samulski et al. (1989). Thus to obtain transduction of 70% of the cells, a multiplicity of up to several hundred vector particles per cell was required. Transduction of human airway epithelial cells in *in vitro* culture using an AAV transducing vector that expressed the cystic fibrosis transmembrane conductance regulator (CFTR) gene from the AAV ITR promoter showed that the cells could be functionally corrected for the electrophysiological defect in chloride channel function that exists in cells from cystic fibrosis patients (Egan et al., Nature, 1992, 358:581-584; Flotte et al., J. Biol. Chem. 268:3781-3790).

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The above-cited studies suggest that AAV vectors have potential utility as vectors for treatment of human disease by gene therapy. However, the difficulty in generating sufficient amounts of AAV vectors has been a severe limitation on the development of human gene therapy using AAV vectors. One aspect of this limitation is that there have been very few studies using AAV vectors in *in vivo* animal models (see, e.g., Flotte et al., 1993b; and Kaplitt et al., 1994, Nature Genetics 8:148-154). This is generally a reflection of the difficulty associated with generating sufficient amounts of AAV vector stocks having a high enough titer to be useful in analyzing *in vivo* delivery and gene expression.

One of the limiting factors for AAV gene therapy has been the relative inefficiency of the vector packaging systems that have been used. In the absence of suitable cell lines expressing sufficient levels of the AAV *trans* complementing functions, such as *rep* and *cap*, packaging of AAV vectors has been achieved in adenovirus-infected cells by co-transfection of a packaging plasmid and a vector. The efficiency of this process is expected to be limited by the efficiency of transfection of each of the plasmid constructs, and by the low level of expression of Rep proteins from the packaging plasmids described to date. Each of these problems appears to relate to the biological activities of the AAV Rep proteins which are known to be associated with pleiotropic inhibitory effects. In addition, as noted above, all of the packaging systems described above have the ability to generate replication-competent AAV by recombination.

The difficulty in generating cell lines stably expressing functional Rep apparently reflects a cytotoxic or cytostatic function of Rep as shown by the inhibition, by Rep protein, of neo-resistant colony formation (Labow et al., 1987; Trempe et al., 1991). This also appears to relate to the tendency of Rep to reverse the immortalized phenotype in cultured cells, which has made the production of cell lines stably expressing functional *rep* extremely difficult. Several attempts to generate cell lines expressing *rep* have been made. Mendelson et al., (1988, Virology, 166:154-165) reported obtaining in one cell line some low level expression of AAV Rep52 protein but no Rep78 or Rep68 protein after stable transfection of Hela or 293 cells with plasmids containing an AAV *rep* gene. Because of the absence of Rep78 and Rep68 proteins, vector could not be produced in the cell line. Another cell line made a barely detectable amount of Rep78 which was nonfunctional.

Vincent et al. (1990, Vaccines 90, Cold Spring Harbor Laboratory Press, pp. 353-359) attempted to generate cell lines containing the AAV *rep* and *cap* genes expressed from the normal AAV promoters, but these attempts were not successful either because the vectors were contaminated with a 100-fold excess of wild-type AAV particles or because the vectors were produced at only very low titers of less than 4×10^3 infectious particles.

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Other variations that have been proposed include systems based on the production of AAV Cap proteins that might be used to reconstitute AAV particles, e.g. by assembly *in vitro* (see, e.g., WO 96/00587, published 01 November 1996); systems employing AAV *rep-cap* genes on a helper virus (see, e.g., WO 95/06743, published 09 March 1995); and systems employing helper viruses from non-human mammals (see, e.g., WO 95/20671, published 03 August 1995).

In yet another approach, Lebkowski et al. (U.S. patent 5,173,414, issued 22 Dec. 1992) constructed cell lines containing AAV vectors in an episomal plasmid. These cell lines could then be infected with adenovirus and transfected with the trans-complementing AAV functions *rep* and *cap* to generate preparations of AAV vector. It is claimed that this allows higher titers of AAV stocks to be produced. However, in the examples shown, the only information relative to titer that is shown is that one human cell line, K562, could be transduced at efficiencies of only 1% or less, which does not indicate high titer production of any AAV vector. In this system the vector is carried as an episomal (unintegrated) construct, and it is stated that integrated copies of the vector are not preferred. In a subsequent patent (U.S. No. 5,354,678, issued 11 Oct. 1994), Lebkowski et al. introduce *rep* and *cap* genes into the cell genome but the method again requires the use of episomal AAV transducing vectors comprising an Epstein-Barr virus nuclear antigen (EBNA) gene and an Epstein-Barr virus latent origin of replication; and, again, the only information relative to titer indicated that it was fairly low.

The approach to packaging of rAAV vectors described by Lebkowski et al., 1992, can be undesirable in several ways. First, maintaining the rAAV vector as an unintegrated, high copy number episomal plasmid in a cell line is not desirable because the copy number per cell cannot be rigorously controlled and episomal DNA is much more likely to undergo rearrangement leading to production of defective vectors. Secondly, in this system, the vector must still be packaged by infecting the cell line with adenovirus and introducing a plasmid containing the AAV *rep* and *cap* genes. The plasmid used by Lebkowski et al. (1992) was again pBal A, which has overlapping homology with the vector ITR sequences and can result in generation of replication-competent AAV. Third, in the pBal A packaging plasmid used by Lebkowski et al., 1988, 1992, the *rep* gene is expressed from its homologous p5 promoter and, since *rep* is generally negatively autoregulated, this would tend to limit *rep* expression.

The problem of suboptimal levels of *rep* expression after plasmid transfection thus also relates to another biological activity of these proteins. There is evidence (Tratschin et al., 1986, Mol. Cell. Biol. 6:2884-2894) that AAV Rep proteins down-regulate their own expression from the AAV-p5 promoter which has been used in the various previously described packaging constructs such as pAAV/Ad (Samulski et al., 1989) or pBal A (Lebkowski et al., 1988, 1992).

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Another attempt to develop cell lines expressing functional *rep* activity was recently published by Hölscher et al. (1994, J. Virol. 68:7169-7177). They described the generation of cell lines in which *rep* was placed under control of a glucocorticoid-responsive MMTV promoter. Although they observed particle formation, the particles were apparently noninfectious. Additional experiments indicated that the defect was quite fundamental; namely, there was virtually no accumulation of single-stranded rAAV DNA in the cells. Production of infectious particles required an additional transient transfection with constitutive highly-expressed *rep* constructs (i.e. they had to "add back" Rep activity to cells that were supposed to be able to provide it themselves). Several other approaches to generating AAV packaging cell lines have also been described recently, see, e.g., T. Flotte et al., WO 95/13365 (Targeted Genetics Corporation and Johns Hopkins University); J. Trempe et al., WO 95/13392 (Medical College of Ohio); and J. Allen, WO 96/17947 (Targeted Genetics Corporation).

There is a significant need for methods that can be used to efficiently generate rAAV vectors that are essentially free of wild-type or other replication-competent AAV; and a corresponding need for cell lines that can be used to effectively generate such rAAV vectors.

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Summary of the Invention

One of the basic challenges for gene therapy has been the development of strategies for transduction of cells and tissues which cannot bc easily manipulated *ex vivo* or which are not actively dividing. AAV vectors can achieve *in vivo* gene transfer, in the respiratory tract for example, but high titers are critical to allow the delivery of a sufficiently high multiplicity of vector in as small a volume as possible. Another important objective is the generation of recombinant AAV (rAAV) vector particles that are substantially free of replication-competent viral particles. Optimal AAV packaging methodology is of central importance in satisfying those objectives and promoting the effectiveness of AAV-based gene therapy. The generation of AAV packaging cell lincs that are both stable and efficient has been elusive, mainly due to the activities of Rep proteins, which tend to down-regulate their own expression (particularly in the absence of helper virs functions) and can negatively affect the host cell. The approaches described herein effectively circumvent these problems and have allowed for substantial improvements in AAV packaging.

The present invention provides recombinasc-activatable AAV packaging cassettes, and packaging cell lines comprising such cassettes, which are useful for generating recombinant AAV particles that mediate delivery of genes of interest to mammalian cells. In the context of the present invention, an AAV packaging gene is "activatably linked" to a promoter based on thc arrangement and relative orientation of various cassette components including first and second site-specific recombination (ssr) sites and an ssr-intervening sequence. The recombinase-activatable AAV packaging cassette is introduced into a mammalian cell to be used for packaging of recombinant AAV vector particles. In the recombinase-activatable state, the arrangement and orientation of elements in the cassette (including a promoter, a first ssr sitc, an ssr-intervening sequence, a second ssr site, and a packaging gene) prevents operable linkage of the promoter and the AAV packaging gene. Activation can be triggered by introducing into the cell and/or causing the expression therein of a gene encoding a recombinase capable of mediating recombination between the first and second ssr sites. As a result of the arrangement and relative orientation of the components within the AAV packaging cassette, the recombination event catalyzed by the recombinase causes the cassette to be re-arranged in a manner that places the AAV packaging gcnc into operable linkage with a promoter. Expression of the AAV packaging gene, which can be deleterious to the host cell, is effectively suppressed but is recombinase-activatable. When the AAV cassettes of the present invention are employed in a mammalian cell comprising a recombinant AAV (rAAV) vector which includes a gene of interest, the gene product(s) of thc

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AAV packaging gene, in conjunction with functions provided by an AAV helper virus, mediate replication and encapsidation of the rAAV vector, resulting in the production of a large number of infectious but replication-incompetent rAAV particles that can be used to deliver the gene of interest to a mammalian target cell.

Various illustrative embodiments of the present invention, including various preferred embodiments, are described in more detail below.

Brief Description of the Drawings

Figure 1 shows the results of Southern blots indicating that recombinase-activatable AAV packaging cassettes of the present invention can be activated by plasmid-encoded recombinases (with or without nuclear localization signals) and used to provide AAV replication functions effective for the rescue and amplification of a stably-integrated rAAV pro-vector, as described in Example 12.

Figure 2 shows the results of Southern blots indicating that recombinase-activatable AAV packaging cassettes of the present invention can be activated by helper-virus-encoded recombinase and used to provide AAV replication functions effective for the rescue and amplification of a stably-integrated rAAV pro-vector, as described in Examples 13 and 15.

Figure 3 shows the results of Southern blots indicating that recombinase-activatable AAV packaging cassettes of the present invention can be activated by recombinase and used to provide AAV replication functions effective for the production of heat-stable infectious AAV vector particles useful for delivering a transgene to a target cell, as described in Example 16.

DETAILED DESCRIPTION OF THE INVENTION

Recombinant AAV vectors are potentially powerful tools for human gene therapy. A major advantage of rAAV vectors over other approaches to gene therapy is that they generally do not require ongoing replication of the target cell in order to become stably integrated into the host cell. The invention described herein provides methods and materials for use in the production of high titers of recombinant AAV vectors for use in gene therapy.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); Oligonucleotide Synthesis (M.J. Gait Ed., 1984); Animal Cell Culture

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(R.I. Freshney, Ed., 1987); the series Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos eds. 1987); Handbook of Experimental Immunology, (D.M. Weir and C.C. Blackwell, Eds.); Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl, eds., 1987); and Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober, eds., 1991). All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

Definitions:

The terms "polypeptide", "peptide" and "protein" are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include, but are not limited to, glycosylation, acetylation and phosphorylation.

"Polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. This term refers only to the primary structure of the molecule. Thus, double- and single-stranded DNA, as well as double- and single-stranded RNA are included. It also includes modified polynucleotides such as methylated or capped polynucleotides.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

A "transcriptional regulatory sequence" or "TRS", as used herein, refers to a genomic region that controls the transcription of a gene or coding sequence to which it is operably linked. Transcriptional regulatory sequences of use in the present invention generally include at least one transcriptional promoter and may also include one or more enhancers and/or terminators of transcription.

"Operably linked" refers to an arrangement of two or more components, wherein the components so described are in a relationship permitting them to function in a coordinated manner. By way of illustration, a transcriptional regulatory sequence or a promoter is operably linked to a coding sequence if the TRS or promoter promotes transcription of the coding sequence. An operably linked TRS is generally joined *in cis* with the coding sequence, but it is not necessarily directly adjacent to it.

"Recombinant," refers to a genetic entity distinct from that generally found in nature. As applied to a polynucleotide or gene, this means that the polynucleotide is the product of various

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combinations of cloning, restriction and/or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a TRS (Transcriptional Regulatory Sequence) or promoter that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous TRS or promoter.

"Sequence overlap" between two polynucleotides occurs when the nucleotides share a homologous sequence. When this homologous sequence is of sufficient length and identity, recombination is facilitated. The level of homology and corresponding frequency of recombination increase with increasing length of the homologous sequences and with their level of shared identity. The level of homology that may pose a concern in a given system can be determined theoretically and confirmed experimentally, as is known in the art. For example, homologous recombination can be substantially reduced or eliminated if the two sequences do not share any stretch of at least 10 base pairs (bp) that is greater than 80% homologous (summed over its length), or any stretch of at least 20 bp that is at least 70% homologous, or any stretch of at least 50 bp that is at least 50% homologous, or any stretch of at least 100 bp that is at least 40% homologous; preferably the levels of homology are even less (preferably less than half of the stated levels), and preferably the lengths of partially homologous sequences are also less (preferably less than half the stated lengths).

A "vector", as used herein, refers to a recombinant plasmid or virus that comprises a polynucleotide to be delivered into a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered, sometimes referred to as a "target polynucleotide" or "transgene", may comprise a coding sequence of interest in gene therapy (such as a gene encoding a protein of therapeutic interest) and/or a selectable or detectable marker.

A "replicon" refers to a polynucleotide comprising an origin of replication which allows for replication of the polynucleotide in an appropriate host cell. Examples of replicons include episomes (including plasmids), as well as chromosomes (such as the nuclear or mitochondrial chromosomes). "Stable integration" of a polynucleotide into a cell means that the polynucleotide has been integrated into a replicon that tends to be stably maintained in the cell. Although episomes such as plasmids can sometimes be maintained for many generations, genetic material carried episomally is generally more susceptible to loss than chromosomally-integrated material. However, maintenance of a polynucleotide can often be effected by incorporating a selectable

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marker into or adjacent to a polynucleotide, and then maintaining cells carrying the polynucleotide under selective pressure. In some cases, sequences cannot be effectively maintained stably unless they have become integrated into a chromosome; and, therefore, selection for retention of a sequence comprising a selectable marker can result in the selection of cells in which the marker has become stably-integrated into a chromosome. Antibiotic resistance genes can be conveniently employed in that regard, as is well known in the art. Typically, stably-integrated polynucleotides would be expected to be maintained on average for at least about twenty generations, preferably at least about one hundred generations, still more preferably they would be maintained permanently. The chromatin structure of eukaryotic chromosomes can influence the level of expression of an integrated polynucleotide. Having the genes carried on episomes can be particularly useful where it is desired to have multiple stably-maintained copies of a particular gene. The selection of stable cell lines having properties that are particularly desirable in the context of the present invention are described and illustrated below.

"AAV" is adeno-associated virus. Adeno-associated virus is a defective parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. General reviews of AAV may be found in, for example, Carter, 1989, Handbook of Parvoviruses, Vol. 1, pp. 169-228, and Berns, 1990, Virology, pp. 1743-1764, Raven Press, (New York). The AAV2 serotype was used in some of the illustrations of the present invention in the Examples. However, it is fully expected that these same principles will be applicable to other AAV serotypes since it is now known that the various serotypes are quite closely related - both functionally and structurally, even at the genetic level (see, e.g., Blacklow, 1988, pp. 165-174 of Parvoviruses and Human Disease, J.R. Pattison (ed); and Rose, 1974, Comprehensive Virology 3: 1-61). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous *rep* genes; and all bear three related capsid proteins such as those expressed in AAV2. The degree of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to ITRs. The similar infectivity patterns also suggest that the replication functions in each serotype are under similar regulatory control.

A "recombinant AAV vector" (or "rAAV vector") refers to a vector comprising one or more polynucleotides of interest (or "transgenes") that are flanked by AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus and is expressing AAV *rep* and *cap* gene products (i.e. AAV Rep and Cap proteins). When an rAAV

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vector is incorporated into a larger polynucleotide (e.g. in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector is typically referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and necessary helper functions).

A "helper virus" for AAV refers to a virus that allows AAV (which is a "defective" parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses and poxviruses such as vaccinia. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and available from depositories such as the ATCC. Viruses of the herpes family include, for example, herpes simplex viruses (HSV) and Epstein-Barr viruses (EBV), as well as cytomegaloviruses (CMV) and pseudorabies viruses (PRV); which are also available from depositories such as ATCC.

"Packaging" as used herein refers to a series of subcellular events that results in the assembly and encapsidation of a viral vector, particularly an AAV vector. Thus, when a suitable vector is introduced into a packaging cell line under appropriate conditions, it can be assembled into a viral particle. Functions associated with packaging of viral vectors, particularly AAV vectors, are described herein and in the art.

AAV "*rep*" and "*cap*" genes are genes encoding replication and encapsidation proteins, respectively. AAV *rep* and *cap* genes have been found in all AAV serotypes examined, and are described herein and in the references cited. In wild-type AAV, the *rep* and *cap* genes are generally found adjacent to each other in the viral genome (i.e. they are "coupled" together as adjoining or overlapping transcriptional units), and they are generally conserved among AAV serotypes. AAV *rep* and *cap* genes are also individually and collectively referred to herein as "AAV packaging genes." Modified AAV packaging genes (including modified *rep* genes and modified *cap* genes) are described below for use in the present invention.

An "AAV split-packaging gene" refers to a recombinant gene encoding one or more AAV packaging proteins (including AAV Rep proteins and/or AAV Cap proteins) wherein the split-packaging gene has been separated from one or more AAV packaging genes to which it is normally linked in the AAV genome. Examples of such AAV split-packaging genes include AAV *split-cap* genes, AAV *rep78* genes and AAV *rep52* genes, as described herein. In preferred embodiments of the present invention, one of more AAV split-packaging genes is "activatably linked" to a promoter, as described below.

An AAV "*split-cap*" gene refers to a recombinant gene encoding one or more AAV Cap

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proteins, which gene is separated from Rep78-specific sequences of an AAV *rep* gene, but is operably linked to a promoter, preferably a heterologous promoter. As described above and in the cited literature, the wild-type AAV *rep* gene contains two promoter sequences, p5 and p19, which drive expression of the "large Rep proteins" (Rep78 and Rep68) and the "small Rep proteins" (Rep52 and Rep40), respectively; and the wild-type AAV *cap* gene contains a promoter, p40, which drives expression of the AAV Cap proteins (VP1, VP2 and VP3). In certain recombinant constructs and cell lines of the present invention, the Cap proteins are encoded by a *split-cap* gene which, unlike the situation in wild-type AAV, is not located next to a gene capable of encoding the large Rep proteins. Rather, an AAV "*rep78*" gene (a gene capable of encoding the large Rep proteins Rep78 and Rep68) is provided separately, at a different location. The *split-cap* genes can be physically separated from Rep78-specific sequences by being present on different replicons or vectors, or, if present on a single replicon or vector, by being separated from Rep78-specific sequences by intervening non-AAV DNA, as described and illustrated in more detail below. In certain preferred embodiments of the present invention, a *split-cap* gene is operably or activatably linked to a heterologous promoter (i.e. a promoter other than the AAV p40 promoter) which heterologous promoter is incorporated upstream of the *split-cap* coding region, either in place of or in addition to the p40 promoter. Examples of such recombinant *split-cap* genes operably or activatably linked to various promoters, including both inducible and constitutive promoters, are described and illustrated below.

An AAV "*rep78*" gene is a gene capable of encoding the large Rep proteins Rep78 and Rep68 (and will generally also encode the small Rep proteins Rep52 and Rep40). In certain preferred embodiments of the present invention, a *rep78* gene is operably or activatably linked to a heterologous promoter (i.e. a promoter other than the AAV p5 promoter) which heterologous promoter is incorporated upstream of the *rep78* coding region, either in place of or in addition to the p5 promoter. Most preferably, the *rep78* gene is activatably linked to a promoter, as described below.

An AAV "*rep52* gene" is capable of separately encoding just the small Rep proteins (i.e. Rep52 and Rep40). Thus, a *rep52* gene may be operably or activatably linked to a promoter (e.g. the p19 promoter or a heterologous promoter in place of or in addition to the AAV p19 promoter). An AAV *split-cap* gene, *rep52* gene and/or *rep78* gene can also be operably or activatably linked to other transcriptional regulatory sequences, including enhancers and polyadenylation ("polyA") sequences (which additional TRS's can also be heterologous). AAV-derived *split-cap* genes, *rep52* genes and *rep78* genes as described herein are collectively referred to as "AAV split-packaging genes." Various examples of the construction and use of such AAV split-packaging

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genes are described and illustrated below.

A "recombinase-activatable AAV packaging cassette" (or simply an "AAV packaging cassette") refers to a polynucleotide comprising the following components in the relative order listed from upstream to downstream: (i) a first site-specific recombination (ssr) site; (ii) an ssr-intervening sequence; and (iii) a second site-specific recombination (ssr) site; wherein the cassette comprises a promoter and an AAV packaging gene selected from the group consisting of an AAV *rep* gene and an AAV *cap* gene, wherein the AAV packaging gene is located either within the ssr-intervening sequence or downstream of the second ssr site, and wherein the promoter is located either within the ssr-intervening sequence or upstream of the first ssr site, and the promoter is activatably linked to the AAV packaging gene. The arrangement and relative orientation of various elements within the cassettes of the present invention results in the generation of a polynucleotide sequence from which the AAV packaging gene is not generally expressed until activation by a recombinase enzyme, which activation places the AAV packaging gene into operable linkage with a promoter, thereby resulting in expression of the AAV packaging gene. Illustrative examples of recombinase-activatable AAV packaging cassettes of the present invention are provided and described in detail below.

A promoter is "activatably linked" to an AAV packaging gene within the AAV packaging cassettes of the present invention by being positioned, relative to the packaging gene and any intervening sequence, such that rearrangement of the ssr-intervening sequence (mediated by recombinase acting at the ssr sites) causes the promoter to become operably linked to the AAV packaging gene. Promoters that are activatably linked to an AAV packaging gene in the context of the present invention thus do not effectively promote transcription of the AAV packaging gene until the corresponding recombinase is introduced (and/or expressed). Examples of such constructs are described and illustrated below.

A "terminator" refers to a polynucleotide sequence that tends to diminish or prevent read-through transcription (i.e. it diminishes or prevents transcription originating on one side of the terminator from continuing through to the other side of the terminator). The degree to which transcription is disrupted is typically a function of the base sequence and/or the length of the terminator sequence. In particular, as is well known in numerous molecular biological systems, particular DNA sequences, generally referred to as "transcriptional termination sequences" are specific sequences that tend to disrupt read-through transcription by RNA polymerase, presumably by causing the RNA polymerase molecule to stop and/or disengage from the DNA being transcribed. Typical examples of such sequence-specific terminators include polyadenylation ("polyA") sequences, e.g., SV40 polyA. In addition to or in place of such

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sequence-specific terminators, insertions of relatively long DNA sequences between a promoter and a coding region also tend to disrupt transcription of the coding region, generally in proportion to the length of the intervening sequence. This effect presumably arises because there is always some tendency for an RNA polymerase molecule to become disengaged from the DNA being transcribed, and increasing the length of the sequence to be traversed before reaching the coding region would generally increase the likelihood that disengagement would occur before transcription of the coding region was completed or possibly even initiated. Terminators may thus prevent transcription from only one direction ("uni-directional" terminators) or from both directions ("bi-directional" terminators), and may be comprised of sequence-specific termination sequences or sequence-non-specific terminators or both. A variety of such terminator sequences are known in the art; and illustrative uses of such sequences within the context of the present invention are provided below.

A "site-specific recombination site" or "ssr site" is a polynucleotide sequence that is recognized and acted upon by a site-specific recombinase enzyme, many of which are known to the art and described herein. Pairs of ssr sites surrounding an ssr-intervening sequence are employed in the context of the AAV packaging cassettes of the present invention. The recombinase acting at the ssr sites mediates an intramolecular DNA rearrangement event, causing either excision or inversion of the ssr-intervening sequence, depending on whether the ssr sites are arranged in tandem or inverted orientation, respectively, as illustrated below.

A "recombinase" refers to a protein that recognizes particular site-specific recombination sites in DNA (termed "ssr sites") and that mediates recombination via those sites. In the context of the present invention, the recombinase recognizes and mediates recombination at corresponding ssr sites incorporated on either side of an ssr-intervening sequence. Illustrative recombinases are described in more detail below and in the art.

An "ssr-intervening sequence" refers to a segment of DNA lying between a pair of ssr sites. The ssr-intervening sequence may include a promoter, a terminator, a selectable marker gene, a packaging gene, a polyA signal, or any other DNA segment lying between a pair of ssr sites as described in connection with various AAV packaging cassettes exemplified herein.

"Efficiency" when used in describing a cell line refers to certain useful attributes of the line; in particular, the growth rate, and (for packaging cell lines) the number of virus particles produced per cell. "High efficiency packaging" indicates production of at least 100 viral particles per cell, more preferably at least about 200 viral particles per cell, still more preferably at least about 400 viral particles per cell. "High safety packaging" indicates that, of the recombinant AAV viral particles produced, fewer than about 1 in 10^6 are replication-competent AAV viral

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particles, preferably fewer than about 1 in 10^8 are replication-competent, more preferably fewer than about 1 in 10^{10} are replication-competent, still more preferably fewer than about 1 in 10^{12} are replication-competent, most preferably none are replication-competent. Preferred packaging cells of the present invention exhibit combinations of such high efficiency and high safety.

"Host cells", "cell lines", "cell cultures", "packaging cell line" and other such terms denote higher eukaryotic cells, preferably mammalian cells, most preferably human cells, useful in the present invention. These cells can be used as recipients for recombinant vectors, viruses or other transfer polynucleotides, and include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical (in morphology or in genomic complement) to the original parent cell.

A "therapeutic gene", "target polynucleotide", "transgene", "gene of interest" and the like generally refer to a gene or genes to be transferred using a vector. Typically, in the context of the present invention, such genes are located within the rAAV vector (which vector is flanked by inverted terminal repeat (ITR) regions and thus can be replicated and encapsidated into rAAV particles). Target polynucleotides can be used in this invention to generate rAAV vectors for a number of different applications. Such polynucleotides include, but are not limited to: (i) polynucleotides encoding proteins useful in other forms of gene therapy to relieve deficiencies caused by missing, defective or sub-optimal levels of a structural protein or enzyme; (ii) polynucleotides that are transcribed into anti-sense molecules; (iii) polynucleotides that are transcribed into decoys that bind transcription or translation factors; (iv) polynucleotides that encode cellular modulators such as cytokines; (v) polynucleotides that can make recipient cells susceptible to specific drugs, such as the herpes virus thymidine kinase gene; and (vi) polynucleotides for cancer therapy, such as E1A tumor suppressor genes or p53 tumor suppressor genes for the treatment of various cancers. To effect expression of the transgene in a recipient host cell, it is preferably operably linked to a promoter, either its own or a heterologous promoter. A large number of suitable promoters are known in the art, the choice of which depends on the desired level of expression of the target polynucleotide; whether one wants constitutive expression, inducible expression, cell-specific or tissue-specific expression, etc. The rAAV vector may also contain a selectable marker.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

The present invention provides novel AAV packaging cassettes each of which comprises an AAV packaging gene that is "activatably linked" to a promoter. Each AAV packaging cassette also comprises a pair of site-specific recombination (or "ssr") sites, each of which is placed in a

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particular orientation with respect to each other (i.e. in tandem or in inverted orientation) on either side of a portion of the cassette which is referred to as the "ssr-intervening sequence". The ssr-intervening sequence may contain a transcriptional terminator, or it may contain the AAV packaging gene or its promoter, as described in connection with the exemplary embodiments of the invention below. The structural arrangement of the various components within the AAV packaging cassettes of the present invention results in the effective suppression of transcription of the AAV packaging gene(s) in the absence of recombinase; and, conversely, the arrangement and relative orientation of components in the cassette results in the activation of transcription of the AAV packaging gene(s) in the presence of the corresponding recombinase (the activation being the result of specific rearrangements of the AAV packaging cassettes that place the AAV packaging gene into operable linkage with a promoter). Packaging cells for use in accordance with the present invention may have one or more different AAV packaging cassettes (expressing one or more AAV replication or encapsidation gene products). When AAV Rep and Cap proteins are expressed in a packaging cell comprising helper functions (such as those provided by an AAV helper virus like Adenovirus), and a recombinant AAV vector that includes a gene of interest, then the rAAV vector can be replicated and encapsidated into rAAV particles - which can be used to deliver the gene of interest to a target cell.

The present invention provides recombinase-activatable AAV packaging cassettes comprising the following components in the relative order listed from upstream to downstream: (i) a first site-specific recombination (ssr) site; (ii) an ssr-intervening sequence; and (iii) a second site-specific recombination (ssr) site; wherein the cassette comprises a promoter (which is located either within the ssr-intervening sequence or upstream of the first ssr site) and an AAV packaging gene selected from the group consisting of an AAV *rep* gene and an AAV *cap* gene (which is located either within the ssr-intervening sequence or downstream of the second ssr site), in a relative structural arrangement such that the promoter is activatably linked to the AAV packaging gene. Each site-specific recombination (ssr) site is a polynucleotide sequence that is recognized by a recombinase, many of which are known to the art and described herein. A DNA rearrangement event (either inversion or excision, depending on whether the cassette is "inversion-type" or "excision-type," respectively) between the two ssr sites activates the packaging gene by placing it into operable linkage with its promoter.

The following exemplary embodiments (listing cassette components, in order) are illustrative of the AAV packaging cassettes of the present invention:

(1) A promoter, a first ssr site, an ssr-intervening sequence comprising a terminator, a second ssr site, and an AAV packaging gene, where the ssr sites are in tandem orientation relative

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to each other, and the terminator prevents transcription originating at the promoter from transcribing the packaging gene (whereby excision mediated by a recombinase acting at the ssr sites deletes the terminator and operably links the promoter to the AAV packaging gene);

(2) A first ssr site, an ssr-intervening sequence comprising a promoter, a second ssr site, and an AAV packaging gene, where the ssr sites are in inverted orientation relative to each other and the promoter is initially in inverted or divergent orientation relative to the AAV packaging gene (whereby inversion mediated by a recombinase acting at the ssr sites inverts the promoter and operably links the promoter to the AAV packaging gene);

(3) A promoter, a first ssr site, an ssr-intervening sequence comprising a uni-directional terminator sequence, a second ssr site, and an AAV packaging gene, where the ssr sites are in inverted orientation relative to each other, the promoter and the AAV packaging gene are in tandem orientation relative to each other, and the uni-directional terminator blocks transcription only from one direction and is arranged in an orientation to prevent transcription originating at the promoter from transcribing the AAV packaging gene (whereby inversion mediated by a recombinase acting at the ssr sites inverts the uni-directional terminator and operably links the promoter to the packaging gene); and

(4) A promoter, a first ssr site, an ssr-intervening sequence comprising an AAV packaging gene, and a second ssr site, where the ssr sites are in inverted orientation relative to each other and the packaging gene is in inverted orientation relative to the promoter (whereby inversion mediated by a recombinase acting at the ssr sites inverts the packaging gene and operably links the packaging gene to the promoter).

Each AAV packaging cassette is thus arranged such that transcription of the AAV packaging is effectively suppressed in the absence of recombinase; and is effectively activated in the presence of the corresponding recombinase. Examples illustrating the construction, testing and use of such cassettes are provided below.

Embodiment (1) above, which is structurally and spatially arranged such that the cassette is activated by excision (which operably links the promoter to the AAV packaging gene), is referred to as an "excision-type" AAV packaging cassette. Embodiments (2)-(4) above, which are arranged such that the cassette is activated by inversion of the ssr-intervening sequence are referred to as "inversion-type" AAV packaging cassettes.

Whether the cassette is excision-type (as in embodiment (1) above) or inversion-type (as in embodiments (2)-(4) above), the cassette is activated by a recombinase protein which mediates recombination at the ssr sites. For excision-type cassettes, the ssr sites are arranged in tandem orientation and activation by recombinase results in excision the ssr-intervening sequence. For

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inversion-type cassettes, the ssr sites are arranged in inverted orientation and activation by recombinase results in inversion of the ssr-intervening sequence.

In particular, where the ssr sites are arranged in tandem orientation on either side of an ssr-intervening sequence, then the recombinase tends to catalyze intramolecular recombination and excision of the ssr-intervening sequence. In a preferred embodiment of the present invention as in (1) above, the ssr sites are arranged in tandem orientation and the ssr-intervening sequence comprises a terminator. Introduction of the corresponding recombinase mediates excision of the ssr-intervening sequence and places the AAV packaging gene into operable linkage with the promoter, thereby allowing activation of expression of the AAV packaging gene.

If the ssr sites are arranged in inverted orientation (relative to each other) on either side of the ssr-intervening sequence, then the recombinase tends to catalyze intramolecular recombination and inversion of the ssr-intervening sequence. In other embodiments of the present invention, as in (2), (3) and (4) above, the ssr sites are arranged in inverted orientation and the intervening sequence may comprise a promoter, an AAV packaging gene or a uni-directional terminator sequence. The recombinase-activatable cassettes of these latter embodiments of the present invention are "inversion-type" in that introduction of the corresponding recombinase mediates inversion of the ssr-intervening sequence, which again, as with excision-type cassettes, places the packaging gene into operable linkage with the promoter, thereby allowing activation of expression.

EXCISION-TYPE AAV PACKAGING CASSETTES

In certain embodiments of the present invention, such as embodiment (1) above, the AAV packaging cassette can be prepared as a recombinant DNA construct in which the ssr-intervening sequence comprises a transcriptional terminator that effectively disrupts the linkage between the promoter and the AAV packaging gene. The degree to which transcription is disrupted by insertion of an ssr-intervening sequence comprising a terminator is generally dependent on the base sequence and/or the length of the DNA sequence. In particular, as is well known in numerous molecular biological systems, particular DNA sequences, which may be referred to as "transcriptional termination sequences" are specific sequences that tend to disrupt read-through transcription by RNA polymerase molecules, presumably by causing the RNA polymerase molecule to stop and/or disengage from the DNA template being transcribed.

In addition to or in place of such sequence-specific terminators, insertions of relatively long DNA sequences between a promoter and a coding region tend to disrupt transcription of the coding region, generally in proportion to the length of the intervening sequence. This effect

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presumably arises because there is always some tendency for an RNA polymerase molecule to become disengaged from the DNA being transcribed, and increasing the length of the sequence to be traversed before reaching the coding region would generally increase the likelihood that disengagement would occur before transcription of the coding region was completed or possibly even initiated. Terminators may thus prevent transcription from only one direction ("uni-directional" terminators) or from both directions ("bi-directional" terminators), and may be comprised of sequence-specific termination sequences or sequence-non-specific terminators or both. Any of a variety of terminators can be employed in conjunction with embodiment (1) above; whereas embodiment (3) above employs a uni-directional terminator.

Where it is desired that transcriptional read-through is especially low prior to activation (which is particularly true in the case of an AAV *rep* gene because expression of AAV Rep proteins can have deleterious effects as noted above), then the terminator sequence will preferably contain one or more specific transcriptional termination sequences, and may also be lengthened by the inclusion of additional DNA sequence so as to further disrupt transcriptional read-through. Preferred terminator sequences of the present invention for use in embodiment (1) above will thus have transcription termination sequences and, especially in the case of *rep* genes, may have a large DNA sequence followed by a transcription termination sequence. Conveniently, such terminator sequences may be comprised of a gene that is followed by a transcription termination sequence, either its own termination sequence or a heterologous termination sequence. Examples of such termination sequences, including various polyadenylation sequences are known in the art and widely available. The use of such terminators is also illustrated below. Where the terminator is comprised of a gene, it can be advantageous to use a gene which encodes a detectable or selectable marker; thereby providing a means by which the presence and/or absence of the terminator sequence (and therefore the corresponding inactivation and/or activation of the cassette) can be detected and/or selected.

Recombinase-activatable AAV packaging cassettes of the present invention can also be flanked by terminators if desired to reduce the possibility that transcriptional activity originating outside of the cassette might influence transcription of the cassette.

INVERSION-TYPE AAV PACKAGING CASSETTES

In other embodiments of the present invention (e.g., embodiments (2)-(4) above), the ssr sites are arranged in an inverted orientation and the cassette is activated by inversion of the ssr-intervening sequence. A first exemplary inversion-type AAV packaging cassette of the present invention (further illustrated below) comprises in order a first ssr site, an ssr-intervening sequence

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comprising a promoter, a second ssr site, and an AAV packaging gene, where the ssr sites are in inverted orientation relative to each other and the promoter is initially in divergent orientation relative to the AAV packaging gene (whereby inversion mediated by a recombinase acting at the ssr sites inverts the promoter and operably links the promoter to the AAV packaging gene). The cassette is introduced into suitable host cells, in which a recombinase is later introduced or expressed. Inversion mediated by a recombinase at the ssr sites inverts the promoter and operably links the promoter to the packaging gene. In this embodiment, no terminator is absolutely required for the cassette. However, to further suppress any potential expression of the packaging gene prior to activation, a terminator may be placed upstream of the first ssr site. This upstream terminator would not be expected to interfere with the recombinase-activatable nature of the cassette, but should prevent undesirable exogenous transcription originating outside the cassette from promoting any transcription of the packaging gene.

A second exemplary inversion-type AAV packaging cassette of the present invention (further illustrated below) comprises in order a promoter, a first ssr site, an ssr-intervening sequence comprising a uni-directional terminator sequence, a second ssr site, and an AAV packaging gene, where the ssr sites are in inverted orientation relative to each other, the promoter and the AAV packaging gene are in tandem orientation relative to each other, and the uni-directional terminator blocks transcription only from one direction and is arranged in an orientation to prevent transcription originating at the promoter from transcribing the AAV packaging gene (whereby inversion mediated by a recombinase acting at the ssr sites inverts the uni-directional terminator and operably links the promoter to the packaging gene). In this embodiment, the ssr sites are inverted relative to each other and the uni-directional terminator blocks transcription only from one direction and is initially arranged in an orientation to prevent transcription originating at the promoter from transcribing the packaging gene. The cassette is introduced into suitable host cells, in which a recombinase is later introduced or expressed. Inversion mediated by a recombinase at the ssr sites inverts the uni-directional terminator and operably links the promoter to the packaging gene. The uni-directional terminator is a terminator which blocks incoming transcription only from one direction.

A third exemplary inversion-type AAV packaging cassette of the present invention (further illustrated below) comprises in order a promoter, a first ssr site, an ssr-intervening sequence comprising an AAV packaging gene, and a second ssr site, where the ssr sites are in inverted orientation relative to each other and the packaging gene is in inverted orientation relative to the promoter (whereby inversion mediated by a recombinase acting at the ssr sites inverts the packaging gene and operably links the packaging gene to the promoter). In this

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embodiment, the promoter is oriented so as to prevent transcription of the ssr-intervening sequence, but the AAV packaging gene located within that sequence is in an inverted orientation relative to the promoter. Any transcription of the ssr-intervening sequence that does occur prior to activation would thus be expected to result in "anti-sense" transcripts (which might be helpful for further suppressing expression of AAV packaging gene functions in the illustrated state). Preferably, in the embodiments of this type, the AAV packaging cassette further comprises a transcriptional terminator located downstream of the second ssr site, and such a terminator should not be expected to interfere with the recombinase-activatable nature of the cassette, but should prevent undesirable exogenous transcription originating outside the cassette from promoting any transcription of the packaging gene.

ACTIVATION OF THE CASSETTES BY RECOMBINASE

If the ssr sites are tandemly (co-directionally) arranged, then the recombinase tends to catalyze intra-molecular recombination and excision of the ssr-intervening sequence (i.e. the sequence located in-between the pair of ssr sites). If the ssr sites are arranged in an inverted orientation, then the recombinase tends to catalyze inversion of the ssr-intervening sequence. The genes and cassettes of the present invention are "recombinase-activatable" in that introduction of the corresponding recombinase mediates excision of an ssr-intervening sequence comprising a terminator sequence or inversion of a ssr-intervening sequence comprising a promoter, an AAV packaging gene, or a uni-directional terminator. In all cases, the cassettes are arranged such that activation by recombinase (via excision or inversion) places an AAV packaging gene into operable linkage with a promoter, allowing activation of expression.

The recombinase can be contacted with the AAV packaging cassette in any of a variety of ways in which proteins can be caused to be expressed in cells. In a particularly convenient method for introducing the recombinase into an AAV packaging cell (which cell might have, for example, a stably integrated AAV packaging cassette), a gene encoding the recombinase can be introduced into the AAV packaging cell with the helper virus necessary to trigger AAV replication/encapsidation. For example, an adenovirus vector can be modified to replace a sequence not essential for helper-virus activity with a gene encoding a recombinase, as described and illustrated below. Any other expression vector comprising a recombinase gene could likewise be used to provide for the production of recombinase protein. Alternatively, it may be possible to have the packaging cell contain a gene for a recombinase which is either tightly regulated (e.g., using a tetracycline transactivator system as referred to below), or which produces an inactive form of a recombinase. In the former case, the recombinase gene may be induced by

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introduction into the medium of a factor that induces expression of the recombinase gene (e.g., tetracycline or doxycycline in the Tet system). In the latter case, the host cells carrying the AAV packaging cassette may express an inactive form of recombinase, e.g. one which is temperature-sensitive, which lacks a necessary co-factor, or which has an extraneous amino acid sequence attached thereto. Activation of the inactive form of recombinase might be mediated by shifting to a permissive temperature, or introducing a factor (such as a co-factor) or other condition (such as expressing a proteinase that cleaves off the extraneous amino acid sequence) to effectively activate the recombinase. Providing recombinase activity can thus be mediated by causing the protein to be expressed, or by causing an inactive form of the protein to become enzymatically active.

AAV PACKAGING GENE(S) FOR USE IN THE PRESENT INVENTION

One of the components of the recombinase-activatable AAV packaging cassettes of the present invention is an AAV packaging gene which may be an AAV *rep* or AAV *cap* gene or any variation or combination thereof. Expression of these genes is relatively complex and potentially deleterious to the host cells. Expression of the *rep* gene, mediated by multiple promoters (p5 and p19) and variable RNA splicing, yields large Rep proteins (i.e. Rep78 and Rep68) and small Rep proteins (i.e. Rep52 and Rep40). The *cap* gene partially overlaps the *rep* gene and encodes three separate proteins, whose production is mediated by RNA splicing and alternative translational start sites. The packaging genes suitable for use in AAV packaging cassettes of the present invention include but are not limited to:

(i) an AAV *split-cap* gene, wherein the *cap* gene is separated from Rep78-specific sequences of an AAV *rep* gene;

(ii) an AAV *rep78* gene, separated from cap-specific sequences of an AAV *cap* gene;

(iii) an AAV *rep52* gene, separated from Rep78-specific sequences of an AAV *rep* gene; and

(iv) combined AAV *rep-cap* genes.

Preferably, using a combination of cassettes as in (i) and (ii) above, the *split-cap* and *rep78* genes may be introduced separately into the host cell line (i.e. they are introduced using separate cassettes, and, if stably integrated into the cell, are stably integrated at different locations). An additional cassette comprising a *rep52* gene can also be included as a means of further elevating expression of the small Rep proteins (i.e. Rep52 and Rep40). At least one of the AAV packaging genes (preferably at least an AAV *rep78* gene) is placed under the control of a recombinase-activatable AAV packaging cassette according the present invention. Other AAV

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packaging genes (such as cap genes and/or *rep52* genes) can also be placed under the control of a recombinase-activatable AAV packaging cassette; or the expression of these can be controlled in another manner. In presently preferred embodiments, however, all AAV packaging genes are preferably placed under the control of recombinase-activatable AAV packaging cassettes. Similarly, using a combination of cassettes as in (i) and (iii) above, the *split-cap* and *rep52* genes may be introduced separately into the host cell line (i.e. they are introduced using separate cassettes, and, if stably integrated into the cell, are stably integrated at different locations). Cassettes encoding various *rep* and/or *split-cap* genes may be introduced into the host cells simultaneously or sequentially, as described and illustrated below. Alternately, as in (iv) above, the *rep* and *cap* genes may be introduced together on a common cassette in which at least the p5 promoter is preferably deleted, and the *rep* gene placed under the control of the recombinase-activatable AAV packaging cassette to avoid potentially deleterious effects of the large Rep proteins. The cassettes may be different types; e.g. one or more may be excision-type or inversion-type, or one may be excision-type while another is inversion-type. It is particularly convenient for all such AAV packaging cassettes in a given cell to be activated by the same recombinase protein (thereby providing concerted activation of all AAV packaging cassettes using a single recombinase).

AAV packaging gene sequences are "separated" or "split" as referred to herein when they are located on different replicons or recombinant DNA constructs or, if present on a single replicon or recombinant DNA construct, when intervening non-AAV DNA sequences is present between them. Typically, they are separated by at least about 100 nucleotides of other (i.e. non-AAV) DNA, more typically by at least about 400 nucleotides, still more typically by at least about 1000 nucleotides (1 kb). The term "non-AAV" DNA refers to sequences that are not substantially homologous to AAV DNA along their length. As is well known and empirically determinable, the low level of substantial homology necessary to essentially avoid homologous recombination events varies in relation to the length of potentially homologous sequences and their level of sequence homology. See, e.g., the publication by Senpathy and Carter, supra, which investigated the degree of such recombination in AAV systems. As a general matter, it will be preferable for the intervening non-AAV sequences to share less than about 20% identity (over stretches of at least about 100 nucleotides) with AAV sequences. More preferably, such sequences should share less than about 10% identity (over stretches of at least about 100 nucleotides); still more preferably, such sequences should share less than about 5% identity (over stretches of at least about 100 nucleotides). Most preferably, such sequences should share less than about 1% identity (over stretches of at least about 100 nucleotides).

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In certain preferred embodiments of the present invention, one or more of the AAV packaging cassettes is stably maintained in the packaging cell line (by integration into a chromosome or a stably-maintained episome); more preferably, at least two of the AAV packaging cassettes are stably maintained in the packaging cell line; most preferably all of the AAV packaging genes are stably maintained in AAV packaging cassettes in the packaging cell line. Such stable maintenance can be mediated by integration of the cassette(s) into one or more stably-maintained episomes or into a chromosome of the cell. Cassettes comprising cap and *rep* genes are preferably maintained on different replicons or, if both are chromosomally integrated, they are preferably introduced separately into the packaging cell line such that they are likely to become integrated at separate sites in the genome. Confirmation that AAV packaging cassettes have become integrated into a packaging cell can be obtained using routine molecular biological techniques (such as Southern blotting with probes directed to each of the separate genes).

As noted above, certain preferred embodiments of the present invention will also comprise a *rep52* gene. Preferably, such a *rep52* gene will also be stably maintained in the packaging cell. The *rep52* gene will preferably be introduced separately from the *rep78* gene but may be introduced together with (or separately from) the *split-cap* gene, as discussed above.

In the preferred embodiments of the present invention, the closely-coupled and tightly-regulated *rep* and *cap* functions characteristic of wild-type AAV, and various prior packaging systems, are thus separated and substantially reorganized in the packaging cell lines of the present invention. The redesigned system of the present invention is useful for providing packaging cell lines for the generation of recombinant AAV vector particles, while at the same time greatly reducing the possibility of generating replication-competent AAV particles.

PROMOTERS FOR USE IN THE AAV PACKAGING CASSETTES

Since transcription of the AAV packaging genes in the cassettes of the present invention can be effectively controlled within the cassette (thereby obviating the potential problems that can arise from expression of proteins such as the Rep proteins in the packaging cell line prior to actual packaging), it is possible to use any of a variety of promoters to effectuate expression of the AAV packaging genes. Thus, even strong and normally constitutive promoters can be employed since transcription of the AAV packaging gene(s) can be effectively suppressed in the recombinase-activatable cassette until the desired activation by a recombinase (which places the promoter into operable linkage with the AAV packaging gene). It will also be possible to employ one or more of the native AAV promoters (such as p5, p19 and/or p40), as well as other heterologous promoters. Combinations of promoters and/or other transcriptional regulatory sequences such as

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enhancers can also be employed.

Since, in certain preferred embodiments, the various *rep* and/or *cap* genes of the present invention may be stably integrated into the host cell genome, location effects (such as those due to chromatin structure) can also influence expression of the genes. As noted above, the cassettes of the present invention can be further "insulated" from extraneous transcriptional activity (which might arise, for example, when an cassette becomes integrated downstream of a promoter) by including terminator sequences adjacent to the cassette. By way of illustration, in embodiment 2 above, a terminator might be located upstream of the first ssr site to effectively block transcription in the downstream direction. The methodology described below can be used to generate and select packaging cells that exhibit the desired properties.

Numerous promoters are known in the art and are generally available; including not only mammalian promoters but numerous promoters of viruses found to infect mammals (such as the commonly-used pCMV promoter illustrated below). A large variety of inducible promoters are also well known in the art and generally available. The cloning of various promoters and confirmation that promoters exhibit the desired levels of expression can be achieved using standard molecular biological techniques as illustrated below and in the references cited herein.

As an initial test for the expression of various *rep* and/or *cap* genes, cells can be readily screened using immunofluorescence or other standard molecular biological techniques to detect Rep and/or Cap proteins; and confirmation of packaging capabilities and efficiencies can be obtained using functional tests for replication and packaging of incoming rAAV vectors. Suitable techniques are described below and in the art.

CONSTRUCTIONS OF SPLIT PACKAGING GENES

Various AAV split-packaging genes (including *rep78* genes, *rep52* genes and *split-cap* genes) can be prepared as recombinase-activatable AAV packaging cassettes as illustrated in the Examples below. Briefly, coding sequences for AAV packaging genes are generally separated from promoter sequences and are introduced into recombinase-activatable AAV packaging cassettes (comprising pairs of site-specific recombination sequences) in which the AAV packaging genes are activatably linked to promoters; see, e.g. Examples 1-8 below. In the context of the present invention, at least one of the AAV packaging genes (preferably at least the *rep78* gene) is incorporated into a recombinase-activatable AAV packaging cassette as described below. Other AAV packaging genes, preferably AAV split-packaging genes, may also be included. These may also be activatably linked to promoters as described and illustrated below. Alternatively, such packaging genes may be operably linked to promoters. The following

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illustrations provide examples of AAV split-packaging genes that are operably linked to heterologous promoters. Such constructs can be used to provide AAV packaging genes that might be introduced into cell lines of the present invention (e.g. in addition to a recombinase-activatable cassette comprising an AAV *rep78* gene); or, alternatively, such constructs can be used as sources of AAV split-packaging genes that can be incorporated into recombinase-activatable AAV packaging cassettes as described in Examples 1-8 below. Thus, AAV packaging genes for use in the recombinase-activatable AAV packaging cassettes of the present invention include wild-type AAV packaging genes (such as the AAV *rep* and/or *cap* genes) as well as split-packaging genes (such as a *split-cap* gene, *rep78* gene and/or *rep52* gene). Wild-type AAV packaging genes have been described in the art and are generally available from a variety of sources. The following illustrations exemplify the construction of various split-packaging genes operably linked to heterologous promoters.

Illustrative construction of an AAV *split-cap* gene operably linked to a heterologous promoter

As an initial illustration of the construction of a *split-cap* gene operably linked to a heterologous promoter, a plasmid called "CMV-cap" was prepared which comprises a heterologous constitutive promoter operably linked to AAV *cap* sequences, and a heterologous polyadenylation (polyA) signal in the following order and relative orientation (the arrowhead symbols (">") as used herein designate the relative directionality, e.g., of promoter activity, transcription, termination, etc.):

--(pCMV>)-(*split-cap*>)-(polyA>)--.

Essentially, the CMV-*cap* plasmid was constructed using standard molecular biological techniques (as described, e.g., in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc., New York, 1987 and updates) to comprise the following component sections:

(i) a HindIII to BglII fragment containing nucleotides 1881-4496 encoding the capsid gene from the pAV2 plasmid (Laughlin, C.A., et al., Gene 23:65-73 (1983)); which (after partially filling in the HindIII site to generate a unique NheI site) was cloned into the following fragment,

(ii) the NheI to BglII backbone of plasmid "tgCMV-HyTK" (Lupton, S.D., et al., Molecular and Cellular Biology 11:3374-3378 (1991)).

The plasmid backbone consists of the following four components:

(i) the BalI-SstII fragment containing the human CMV IE94 promoter (Boshart, M.F., et al., Cell 41:521-530 (1985));

(ii) nucleotides 1881-4496 from the pAV2 plasmid (Laughlin, C.A., et al., Gene 23:65-

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73 (1983)) encoding the *cap* gene;

(iii) the Bcl-BamHI fragment from the simian virus 40 genome containing the SV40 early region polyA region (Tooze, J. (ed.), Molecular Biology of Tumor Viruses, DNA tumor viruses (2nd ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1981)); and

(iv) the NruI-AlwN1 fragment from pML2d (Lusky, M. and M. Botchan, Nature 293:79-81 (1981)) containing the bacterial replication origin and the AlwNI-AatII fragment from pGEM1 (Promega Corp.) containing the beta-lactamase gene (which provides ampicillin resistance to facilitate the cloning process).

Illustrative construction of an AAV *rep78* gene operably linked to a heterologous promoter

As an illustration of the construction of a *rep78* gene operably linked to a heterologous promoter, a plasmid called "mMT1-rep78" was prepared which comprises a heterologous inducible promoter operably linked to AAV *rep* sequences, and by a heterologous polyA signal in the following order and orientation:

--(mMT1 promoter>)-(*rep78* >)-(polyA>)--.

Essentially, the mMT1-rep78 plasmid was constructed using standard molecular biological techniques (as described, e.g., in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc., New York, 1987 and updates) to comprise the following four component sections:

i) vector backbone: pBluescript KS (+) (available from Stratagene), into which had been inserted a neomycin resistance gene (under the control of an SV40 promoter and followed by an SV40 polyA sequence (as described for pMT-rep/cap//pKO-neo in WO 96/17947, by James M. Allen and Targeted Genetics Corporation, published 13 June 1996) to facilitate selection in both bacterial cells (using neomycin) and in mammalian cells (using G418);

ii) mMT1: mouse metallothionein I regulatory region on a KpnI/BglII fragment (KpnI is at -589 according to Bacolla, A. et al., Nucleic Acids Res. 19:1639-1647, 1991; BglII is +64 according to Glanville et al., Nature 292:267-269, 1981);

iii) *rep*^{78}: AAV *rep* sequences from 311 to 2188 (sequence according to Srivastava et al., J. Virol. 45:555-564, 1983) followed by the sequence 5'-CTAGA CCTCC TCAGA TTAGC GAGGG GCCAT AGCTT ATGAG CTAGC CGC-3' (SEQ ID NO:1), to provide the spliced second *rep* exon; and

iv) polyA signal: from the mouse metallothionein I gene on an SstII/HindIII fragment (SstII is at 925 and HindIII is at 1246 according to Glanville et al., Nature 292:267-269, 1981).

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Illustrative construction of an AAV *rep52* gene

As an illustration of the construction of a *rep52* gene, a plasmid called "mMT1-rep52" was prepared which comprises a heterologous inducible promoter operably linked to AAV *rep52* sequences, and a heterologous polyA signal in the following order and orientation:

--(mMT1 promoter>)-(*rep52* >)-(polyA>)--.

Essentially, plasmid mMT1-rep52 was constructed using standard molecular biological techniques (as described, e.g., in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc., New York, 1987 and updates) to comprise the following four component sections:

i) vector backbone: pBluescript KS (+) (available from Stratagene), into which had been inserted a neomycin resistance gene (under the control of an SV40 promoter and followed by an SV40 polyA sequence (as described for pMT-rep/cap//pKO-neo in WO 96/17947, by James M. Allen and Targeted Genetics Corporation, published 13 June 1996) to facilitate selection in both bacterial cells (using neomycin) and in mammalian cells (using G418);

ii) mMT1: mouse metallothionein I regulatory region on a KpnI/BglII fragment (KpnI is at -589 according to Bacolla, A. et al., Nucleic Acids Res. 19:1639-1647, 1991; BglII is +64 according to Glanville et al., Nature 292:267-269, 1981);

iii) rep^{52}: AAV *rep* sequences from 964 to 2188 (sequence according to Srivastava et al., J. Virol. 45:555-564, 1983) followed by the sequence 5'-CTAGA CCTCC TCAGA TTAGC GAGGG GCCAT AGCTT ATGAG CTAGC CGC-3' (SEQ ID NO:2), to provide the spliced second *rep* exon; and

iv) polyA signal: from the mouse metallothioncin I gene on a SstII/HindIII fragment (SstII is at 925 and HindIII is at 1246 according to Glanville et al., Nature 292:267-269, 1981).

Illustrative construction of a first expression plasmid comprising separated *split-cap* and *rep52* genes (tandem orientation)

As a first illustration of the construction of an expression plasmid comprising a *split-cap* gene and a *rep52* gene, a plasmid called "CMV-cap/mMT1-rep52/version 1 (or tandem orientation)" was prepared which comprises a heterologous constitutive promoter operably linked to AAV *cap* sequences, and a heterologous inducible promoter operably linked to AAV *rep52* sequences, each incorporated into the plasmid in the same transcriptional orientation.

Thus the CMV-cap/mMT1-rep52/version 1 plasmid contains:

--(pCMV>)-(*split-cap*>)-(polyA>)-(mMT1 promotcr>)-(*rep52*>)-(polyA>)--.

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Essentially, plasmid CMV-cap/mMT1-rep52/version 1 was constructed using standard molecular biological techniques (as described, e.g., in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc., New York, 1987 and updates) to comprise the following three component sections:

i) vector backbone: pBluescript KS (+) (as above);

ii) a pCMV-*split-cap* gene (constructed as described above in Sample Construction 1), inserted into an mMT1-rep52 vector (as described above), by insertion at a NotI site in the polylinker 5' of the mMT1 regulatory region such that the pCMV promoter is oriented in the same direction as the mMT1 promoter; and

iii) mMT1-rep52: (as described above).

Illustrative construction of a second expression plasmid comprising *split-cap* and *rep52* genes (divergent orientation)

As an illustration of the construction of a second expression plasmid comprising a *split-cap* gene and a *rep52* gene, a plasmid called "CMV-cap/mMT1-rep52/version 2 (or divergent orientation)" was prepared which comprises a heterologous constitutive promoter operably linked to AAV *cap* sequences, and a heterologous inducible promoter operably linked to AAV *rep52* sequences, incorporated into the plasmid in opposing transcriptional orientations. Thus the CMV-cap/mMT1-rep52/version 2 plasmid contains, in order:

-- (<polyA)-(<*split-cap*)-(< pCMV)-(mMT1 promoter>)-(*rep52*>)-(polyA>)--.

Essentially, CMV-cap/mMT1-rep52/version 2 was constructed using standard molecular biological techniques (as described, e.g., in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc., New York, 1987 and updates) to comprise the following three component sections:

i) vector backbone: pBluescript KS (+) (as above);

ii) a pCMV-*split-cap* gene (constructed as described above), inserted into an mMT1-rep52 vector (as described above), by insertion at a NotI site in the polylinker 5' of the mMT1 regulatory region such that the pCMV promoter is oriented in the opposite direction as the mMT1 promoter; and

iii) mMT1-rep52: (as described above).

RECOMBINASES FOR USE IN THE PRESENT INVENTION

The recombinase is a protein that recognizes particular site-specific recombination sites in DNA (termed "ssr sites") and mediates recombination via those sites. In the context of the

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present invention, the recombinase recognizes and mediates recombination at corresponding ssr sites incorporated on either side of an ssr-intervening sequence. As noted above, the effect of the recombination event depends on whether the ssr sites are arranged in a tandem or inverted fashion, resulting in excision or inversion respectively. By way of illustration, the recombinase from bacteriophage P1 (called "Cre" for cyclization recombination) is a 38 kilodalton protein that specifically recognizes two 34-bp repeats termed loxP sites, each of which comprises two 13-bp inverted repeats flanking an 8-bp core region (see, e.g., Sternberg, N., Cold Spring Harbor Symp. Quant. Biol., 1978, 43:1143-1146; Hoess, R., et al., 1982, Proc. Natl. Acad. Sci. USA, 79: 3398-3402; and Hoess, R., et al., Nucleic Acids and Molecular Biology, 1990, 4:90-109). Similarly, FLP recombinase from the 2 micron plasmid of *Saccharomyces cerevisiae* recognizes sites denoted "FRT" (FLP Recognition Target) sites and mediates recombination between them (see, e.g., Broach, J., et al., Cell, 1982, 29:227-234; Jayaram, M., Trends Biochem. Sci., 1994, 19:78-82; and Fiering, et al., Proc. Natl. Acad. Sci. USA 90: 8469-8473, 1993). A number of other site-specific recombination sequences are known in the art and new site-specific recombinases are being regularly identified in various systems (see, e.g., Craig, N.L., Ann. Rev. Genet., 1988, 22:77-105; Argos, P., et al., EMBO Journal, 1986, 5:433-440; Landy, A., Curr. Opin. Genet. Dev., 1993, 3:699-707; Sadowski, P.D., FASEB Journal, 1993, 7:760-767; and Abremski, K., et al., Protein Eng., 1992, 5:87-91). Genetic methods involving recombination of DNA have also been described (see, e.g., Bebee et al., U.S. Pat. No. 5,434,066; Sauer, U.S. Pat. No. 4,959,317; Backman, U.S. Pat. No. 4,673,640; Srivastava, U.S. Pat. No. 5,252,479; Enquist et al., Eur. Pat No. 0 300 422 B1; Wahl et al., PCT Publ. WO 92/15694; Dzieglewska, PCT Publ. WO 94/19460; Anton and Graham, 1995, J. Virol., 69:4600-4606; Kanegae et al., 1995, Nucleic Acids Res., 23:3816-3821; Wang, 1996, Proc. Natl. Acad. Sci. USA, 93: 3932-3936; Sakai et al., 1995, Biochem. Biophys. Res. Comm., 217: 393-401; Wang et al., 1995, Somatic Cell Mol. Gen., 21:429-441).

The recombinase can be contacted the AAV packaging cassette in any of a variety of ways in which proteins can be caused to be expressed in cells. In a particularly convenient method for introducing the recombinase into an AAV packaging cell (which cell might have, for example, a stably-integrated AAV packaging cassette), a gene encoding a recombinase can be introduced into the AAV packaging cell as part of an AAV helper virus that is used to provide helper functions for AAV replication/encapsidation. Examples of such AAV helper viruses include adenoviruses, herpesviruses, and other viruses as known in the art. For example, an adenovirus vector can be modified to replace a sequence not essential for helper-virus activity (in a particular packaging cell) with a gene encoding a recombinase, as described and illustrated

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below. Any other expression vector comprising a recombinase gene could likewise be used to provide for the production and/or expression of recombinase protein. Alternatively, it may be possible to have the packaging cell contain a gene for a recombinase which is either tightly regulated, or which produces an inactive form of a recombinase. Providing recombinase activity could thus be mediated by causing the protein to be expressed, or by causing the inactive form of the protein to become enzymatically active.

One mechanism that can be used to provide a tightly-controlled recombinase gene (which can then be stably incorporated into the packaging cell) is to place the recombinase gene under the control of a tetracycline transactivator system such as that described in Gossen, M., et al., Science 268:1766-1769, 1995. In preferred embodiments of that type, the recombinase is placed under the control of a "Tet-On" regulatory sequence such that expression of the recombinase gene can be turned on with the addition of tetracycline (or derivatives such as doxycycline) to the cell medium.

PACKAGING CELL LINES

The parental lines from which packaging cells are generated may be obtained from any cell line that is susceptible to AAV infection, and amenable to culture *in vitro*. As indicated above, AAV has a very broad host range and has been isolated from a variety of mammalian cell types, including simian, human and rodent cells. For human gene therapy, human cell lines in which appropriate helper functions can be expressed are typically preferred. Such human cell lines from which the packaging cell lines may be derived, include, for example, Hela, A549, 293, KB, Detroit, and WI38 cells. Various derivatives of human 293 cells and derivatives of Hela cells were initially selected for demonstrations of the present invention. Preferred cell lines include those which are adapted or adaptable to growth in suspension, which can facilitate the use of large-scale vector production techniques (e.g., techniques employing "bioreactors"). By way of illustration, experiments were performed as described below using suspension-adapted cell lines derived from human Hela cells and 293 cells.

A variety of mammalian packaging cell lines, including "universal" packaging cell lines that can be used for the production of a variety of different rAAV vectors, are described below.

GENERATION OF RECOMBINANT AAV PRO-VECTORS, AND PACKAGING OF VECTORS, USING CELLS COMPRISING AAV PACKAGING CASSETTES

The production of rAAV vector particles (i.e. containing an rAAV vector having a transgene of interest encapsidated into an AAV viral particle) requires the presence in a

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susceptible mammalian cell (i.e. a mammalian cell capable of infection and replication of AAV) of the following elements:

(i) an rAAV vector or pro-vector which serves as the template for replication (to generate progeny rAAV vector polynucleotides capable of being encapsidated into AAV particles);

(ii) AAV packaging proteins, i.e. AAV Rep and Cap proteins necessary for catalyzing the replication and encapsidation of AAV pro-vector (in the context of the present invention, one or more of the AAV packaging proteins is encoded by a recombinase-activatable AAV packaging cassette whereby expression of the AAV packaging protein is suppressed until the packaging cassette is activated by recombinase);

(iii) AAV helper functions provided by an AAV helper virus or genes derived therefrom, e.g. an adenovirus or adenovirus genes providing helper activities necessary for AAV replication and/or encapsidation (if a helper virus is used, the helper virus can also carry one or more AAV packaging genes and/or a recombinase gene);

(iv) a recombinase protein (which can be expressed from the cellular genome, from the helper virus, or from another source as described and/or illustrated below).

These elements can be provided on one or more replicons or vectors within a suitable AAV packaging cell.

For example, a "two-component" system can comprise:

(1) a packaging cell comprising an rAAV vector and AAV packaging genes; and

(2) a helper virus providing a recombinase gene as well as helper functions.

If all necessary AAV packaging genes are carried by the packaging cell, it may not be necessary to include any AAV packaging genes on the helper virus; although it may be beneficial to do so to increase expression of a particular gene product. Alternatively, some packaging genes can be carried in the cell and some in the helper virus. Similarly, the rAAV vector can be conveniently provided in the packaging cell, but also can be introduced into a helper virus; it can also be introduced transiently by transfection or can be introduced by infection (see, e.g., the use of such cell lines for "passage packaging" as described below).

One-component systems can also be designed in which all necessary components (including helper virus functions and the recombinase) are incorporated into the packaging cell (episomally or chromosomally). The recombinase gene is then preferably placed under inducible control (such as the tetracycline transactivator system described above), or the recombinase gene product is produced in an inactive form that can be activated by a shift in conditions (as discussed above).

The generation of rAAV-specific packaging cells of the present invention can be obtained

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by infecting a packaging cell with an rAAV vector or transfecting it with an rAAV pro-vector or "vector" comprising, for example, a heterologous transgene, such as a therapeutic gene, flanked by AAV ITR regions. Under suitable conditions (e.g., suitable growth conditions and the presence of helper virus functions), recombinase-activated expression of *rep* and *cap* genes in the packaging cell results in the synthesis of Rep and Cap proteins, which in turn mediate replication and encapsidation of the AAV vector. Providing a polynucleotide of interest (also referred to as a "target polynucleotide" or "transgene") in-between the AAV ITR sequences of the rAAV vector thus results in packaging of the transgene into infectious rAAV particles. These particles may subsequently be used to deliver the transgene to other desired host cells, *ex vivo* or *in vivo*.

Generally, the transgene is operably linked to a promoter, either its own or a heterologous promoter. A large number of suitable promoters are known in the art, the choice of which depends on the desired level of expression of the transgene; whether one wants constitutive expression, inducible expression, cell-specific or tissue-specific expression, etc. An rAAV vector can be initially prepared within a "shuttle vector" such as a plasmid that can be used to facilitate cloning of the rAAV vector.

For example, where the rAAV vector is to contain a transgene operably linked to a heterologous promoter, the transgene can be cloned into a site in the shuttle vector using standard molecular biological techniques such that the transgene is operably linked to a heterologous promoter and the transgene-promoter cassette is flanked by AAV ITR sequences. As is known in the art, selectable marker genes can also be used to facilitate cloning. For example, the transgene can comprise a positive selectable marker (or the shuttle vector can comprise a negative selectable marker that is replaced by the transgene) thus facilitating selection of shuttle vector constructs comprising the transgene insert.

Preferably, the rAAV vector will also contain a positive selectable marker in order to allow for selection of cells that have been infected by the rAAV vector.

As described in the Background section, the presence of contaminating replication-competent AAV particles (i.e. AAV particles not containing the transgene) can limit the therapeutic potential of rAAV vector preparations. The proportion of these undesirable AAV particles can be minimized by decreasing the sequence overlap between the rAAV vector and any AAV genes in the cassettes in the packaging cell line, which decreases the likelihood of homologous recombination. The rAAV pro-vector will preferably comprise at least one and, more preferably, two AAV ITR regions, but will preferably not share any significant sequence overlap with AAV *rep* or *cap* genes. In addition to this lack of significant sequence overlap between the rAAV vector and the AAV packaging genes in preferred embodiments of the present

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invention, the AAV packaging genes are also "split", or separated from each other, thereby further reducing the possibility that a replication-competent AAV particle could be reconstituted by even non-homologous recombination events. The packaging cell lines of the present invention enable the efficient production of rAAV preparations that are of high titer and are substantially free of any contaminating replication-competent AAV; attributes that are especially useful in the context of AAV-mediated gene therapy.

Various transgenes of interest may be introduced into rAAV vectors. Such transgenes include "wild-type" genes encoding proteins of interest, as well as derivatives thereof which encode, for example, proteins that are functionally equivalent to the wild-type, or proteins that share one or more functions of the wild-type. By way of illustration, we have used rAAV vectors containing polynucleotides that encode a functional cystic fibrosis transmembrane conductance regulator polypeptide (CFTR) operably linked to a promoter. As is now known in the art, there are a variety of CFTR polypeptides that are capable of reconstructing CFTR functional deficiencies in cells derived from cystic fibrosis patients. For example, Rich et al. (1991, Science, 253: 205-207) described a CFTR derivative missing amino acid residues 708-835, that was capable of transporting chloride and capable of correcting a naturally occurring CFTR defect. Egan et al. (1993) described another CFTR derivative (comprising about 25 amino acids from an unrelated protein followed by the sequence of native CFTR beginning at residue 119) that was also capable of restoring electrophysiological characteristics of normal CFTR. To take two additional examples, Arispe et al. (1992, Proc. Natl. Acad. Sci. USA 89: 1539-1543) showed that a CFTR fragment comprising residues 433-586 was sufficient to reconstitute a correct chloride channel in lipid bilayers; and Sheppard et al. (1994, Cell 76: 1091-1098) showed that a CFTR polypeptide truncated at residue 836 to about half its length was still capable of building a regulated chloride channel. Thus, the native CFTR protein, and mutants and fragments thereof, all constitute CFTR polypeptides that are useful as exemplary transgenes in the context of the present invention.

Other useful transgenes can be used in this invention to generate rAAV vectors for a number of different applications. Such transgenes include, but are not limited to: (i) polynucleotides encoding proteins useful in other forms of gene therapy to relieve deficiencies caused by missing, defective or sub-optimal levels of a structural protein or enzyme; (ii) polynucleotides that are transcribed into anti-sense molecules; (iii) polynucleotides that are transcribed into decoys that bind transcription or translation factors; (iv) polynucleotides that encode cellular modulators such as cytokines; (v) polynucleotides that can make recipient cells susceptible to specific drugs, such as the herpes virus thymidine kinase gene; and (vi)

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polynucleotides for cancer therapy, such as E1A or p53 tumor suppressor genes.

Some transgenes can provide multiple activities such as those above. For example, an E1A gene (which can be derived from an adenovirus) can be used to both suppress tumorigenesis (and/or metastasis) associated with a number of different cancer cell types, and can also function to render such cells more sensitive to other anti-cancer therapies such as chemotherapy or radiotherapy.

Since the therapeutic specificity of the resulting recombinant AAV vector is determined by the vector introduced, the same basic packaging cell line can be modified for any of these applications. The plasmid comprising the transgene can be introduced into the packaging cell for production of the AAV vector by one of several possible methods; including, for example, electroporation, transfection, lipofection and the like.

The replication and packaging of rAAV vectors in a mammalian host cell generally involves: (i) an rAAV pro-vector, (ii) AAV Rep and Cap proteins, and (iii) helper virus functions necessary to promote AAV replication and packaging. A "helper virus" for AAV production is a virus that provides functions (including, but not limited to, the recombinase gene) necessary to allow the AAV vector to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses and poxviruses such as vaccinia. Helper viruses may be human or non-human in origin. Helper virus or helper virus functions can be introduced before, during or after introduction of the rAAV vector. For example, a plasmid carrying the AAV packaging cassette can be co-introduced into the culture along with the helper virus. Alternatively, helper virus functions can be provided by introducing genes encoding such functions into a packaging cell (either transiently or stably). In either case, after helper functions are provided, the cells can be cultured for a sufficient period, typically 2-5 days, in conditions suitable for replication and packaging as known in the art (see references above and examples below). Lysates can then be prepared, and the recombinant AAV vector particles can be purified by techniques known in the art.

GENERATION OF "UNIVERSAL" AAV PACKAGING CELL LINES COMPRISING RECOMBINASE-ACTIVATABLE AAV PACKAGING CASSETTES

The present invention can be used to generate "universal" AAV packaging cells that can be used to produce any of a variety of rAAV vectors of interest. Such a universal packaging cell might contain, for example, one or more stably integrated AAV packaging genes (preferably within AAV packaging cassettes according to the present invention) which are collectively capable of providing AAV packaging proteins (i.e. AAV Rep and Cap proteins). The remaining

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functions needed for rAAV vector production, i.e. helper virus functions, an rAAV pro-vector and a recombinase, can be provided in any of a variety of ways. By way of illustration, a helper virus such as a recombinant adenovirus, can be used to provide helper virus functions as well as a recombinase and/or an rAAV pro-vector, since (as is known in the art) a number of genes in such a helper virus (e.g. the E3 gene of adenovirus) can be replaced without eliminating helper virus activity. Additional genes can be inserted into such a helper virus by providing any necessary helper virus functions *in trans*. For example, human 293 cells contain adenoviral genes that can complement adenoviral E1 mutants. Thus, heterologous genes can also be cloned into an adenovirus in which the E1 genes have been deleted, for use in cells that can effectively provide such adenoviral functions *in trans*. Alternatively, the use of a helper virus can be eliminated by providing all necessary helper virus functions in the packaging cell. Effective construction of such a packaging cell is feasible in the context of the present invention because the AAV packaging genes (such as *rep* and cap), which are normally up-regulated in the presence of helper virus, can be effectively controlled within the recombinase-activatable AAV packaging cassettes of the present invention. In addition, any adenovirus function that has a deleterious effect on the host packaging cell can likewise be placed under the control of a recombinase-activatable cassette analogous to the recombinase-activatable AAV packaging cassettes described herein. Any other functions needed for rAAV production (e.g. rAAV vector and recombinase) can be provided separately or together, by any of a variety of means, such as transfection, lipofection, electroporation and the like. Such techniques can be used to introduce the genes either stably or transiently, as is known in the art.

Integration of genes into the packaging cells of the present invention can be conveniently monitored using Southern analysis, for example. Expression of Rep and/or Cap proteins can be assayed using any of a variety of techniques; including structural assays (such as "Northern" and/or "Western" blotting), and functional assays (such as replication and packaging of an incoming rAAV vector), as illustrated herein and in the art.

Particularly preferred packaging cells of the present invention produce very little, if any, replication-competent AAV particles; preferably, the frequency of generation of replication-competent AAV particles is less than about 1 per 10^6 particles produced, preferably less than 1 per 10^8, still more preferably less than 1 per 10^{10}, and most preferably, less than 1 per 10^{12}.

Preferred packaging cells of the present invention are also capable of replicating at least one half as rapidly as the parental cells from which they were derived, more preferably at least two-thirds as rapidly, still more preferably at least 90% as rapidly. Preferred packaging cells according to the present invention are also capable of producing at least about 100 rAAV

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particles/cell, more preferably at least about 200 rAAV particles/cell, still more preferably at least about 400 rAAV particles/cell.

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

EXAMPLES

EXAMPLE 1
CONSTRUCTION OF A POLYNUCLEOTIDE COMPRISING A PAIR OF SITE-SPECIFIC RECOMBINATION (SSR) SITES

As an illustrative ssr-recombinase system, we initially used the "loxP" system of bacteriophage P1. The recombinase of bacteriophage P1 (called "Cre" for cyclization recombination) is a 38 kilodalton protein that specifically recognizes two 34-bp repeats termed loxP sites, each of which comprises two 13-bp inverted repeats flanking an 8-bp core region (see, e.g., Sternberg, N., Cold Spring Harbor Symp. Quant. Biol., 1978, 43:1143-1146; Hoess, R., et al., 1982, Proc. Natl. Acad. Sci. USA, 79: 3398-3402; and Hoess, R., et al., Nucleic Acids and Molecular Biology, 1990, 4:90-109).

We synthesized a 112 nucleotide sequence that comprises a pair of tandemly-oriented loxP ssr sites flanked by heterologous DNA that contained restriction endonuclease sites to facilitate subsequent cloning. The 112 nucleotide fragment, termed "loxP-loxP", contained the following sequence:

5'-cgaag gcctA TAACT TCGTA TAGCA TACAT TATAC GAAGT TATgc ggccg cgtcg acATA ACTTC GTATA GCATA CATTA TACGA AGTTA Tagat ctctc gagaa gctta gc-3' (SEQ ID NO:3).

Since the ssr sites (in uppcr case letters) are arranged in tandem orientation, such a polynucleotide can be conveniently used to construct any of a variety of excision-type AAV packaging cassettes of the present invention, as illustrated below.

Inversion-type cassettes of the present invention can be prepared using an analogous ssr-ssr fragment in which the ssr sites are arranged in inverted orientation; e.g. one ssr site (either nucleotides 10 to 43 or nucleotides 58 to 91 in the sequence above) is reversed while the remainder of the sequence is left intact. In the case of loxP, the second loxP site is preferably invertcd such that it faces the first, i.e. the sites are arranged in a convergent orientation with respect to each other.

Other pairs of ssr sites, such as "FRT" sites (for FLP Recognition Target; see, e.g.,

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Broach, J., et al., Cell, 1982, 29:227-234; and Jayaram, M., Trends Biochem. Sci., 1994, 19:78-82) can be generated in an analogous fashion.

EXAMPLE 2

CONSTRUCTION OF A GENERIC VECTOR FOR THE PREPARATION OF A VARIETY OF EXCISION-TYPE AAV PACKAGING CASSETTES OF THE PRESENT INVENTION

Preferred excision-type AAV packaging cassettes of the present invention comprise the following components in the relative order and orientation shown below:

--(promoter>)-(ssr>)-(ssr-intervening sequence)-(ssr>)-(AAV packaging gene>)-(polyA>)--

(the arrowhead symbol (">") is used herein to indicate relative directionality, i.e. of promoter activity, transcription, terminator activity, etc., as well as the relative orientation of the ssr sites with respect to each other).

In preferred embodiments of excision-type AAV packaging cassettes of the present invention, the ssr-intervening sequence comprises a terminator that prevents transcription originating from the upstream promoter from causing transcription of the AAV packaging gene which is located downstream of the second ssr site. Activation of the cassette by recombinase results in excision of the ssr-intervening sequence and, in the rearranged cassette, the downstream AAV packaging gene is placed into operable linkage with the upstream promoter.

Example 2a

Construction of a vector comprising a promoter and tandemly-oriented ssr sites

In this example, we illustrate construction of a generic intermediate vector, called "pCMVloxP-loxP", comprising a constitutive promoter (from CMV, i.e. an intermediate early promoter) and a pair of tandemly-oriented ssr sites, arranged in the relative order and orientation shown below:

--(pCMV>)-(ssr>)-(ssr>)--

The plasmid "pCMVloxP-loxP" was first constructed by subcloning a Stu I/Hind III double stranded DNA oligonucleotide of about 100 bp (from the 112 bp loxP-loxP sequence described in Example 1) into the Stu I/Hind III sites of a 3.4 kb DNA fragment from pCMVβ (commercially available from Clontech). The loxP-loxP oligonucleotide thus contains two loxP sites arranged in tandem, separated by a poly-cloning sequence that includes Not I and Sal I restriction sites. Additional restriction enzyme sites, including a Bgl II and an Xho I site are

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present downstream of the second loxP site. The resulting plasmid can be used to readily introduce an ssr-intervening sequence in-between the ssr sites, and can be used to generate complete excision-type AAV packaging cassettes by cloning AAV packaging genes downstream of the second ssr site (as illustrated in the following examples).

Example 2b

Introduction of an ssr-intervening sequence into the vector

The generic vector of Example 2a was modified by insertion of an ssr-intervening sequence, comprising a selectable marker gene and a transcriptional terminator, into the poly-cloning sequence separating the tandemly-arranged ssr sites. Specifically, a 4.3 kb Not I/Sal I fragment containing the "β-geo" gene (a reporter gene encoding a protein with both β-galactosidase and neomycin phosphotransferase activity, as described in Friedrich, G., et al. Genes & Development 5:1513-1523, 1991) followed by an SV40 polyA was subcloned into the Not I / Sal I sites of pCMVloxP-loxP.

The resulting plasmid, termed pCMVloxP-β-geo-loxP, comprises the following components in the relative order and orientation shown below:

--(pCMV>)(ssr>)(β-geo>)(term>)(ssr>)--

This plasmid can then be used to generate any of a variety of excision-type AAV packaging cassettes by introducing an AAV packaging gene into the position downstream of the second ssr site (e.g., by cloning into one of the restriction endonuclease sites located in the polycloning sequence introduced adjacent to the ssr, as described in Example 1). Illustrative constructions and uses of such cassettes are described below.

EXAMPLE 3

CONSTRUCTION OF AN AAV PACKAGING CASSETTE COMPRISING A REP78 GENE

As a first illustration of a recombinase-activatable AAV packaging cassette of the present invention, we constructed a plasmid called "CMVOFFRep78". A 2.3 kb Bgl II/Xho I DNA fragment containing the AAV *rep* gene (from the plasmid pMt-rep/cap//pKO-neo as described in a commonly-owned application published as WO 96/17947 on 13 June 1996) and an SV40 polyAdenylation (polyA) signal was subcloned into the Bgl II/Xho I sites of pCMVloxP-β-geo-loxP. The resulting plasmid is designated pCMVOFFRep78 and comprises the following components in the relative order and orientation shown below:

--(pCMV>)-(ssr>)-[(β-geo>)-(polyA>)]-(ssr>)-(*rep78*>)-(polyA>)--

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Following activation of the packaging cassette by the corresponding recombinase (e.g., the Cre protein), the ssr-intervening sequence is excised and the rearranged cassette contains the AAV packaging gene in operable linkage with the promoter, as follows:

--(pCMV>)-(ssr)-(*rep78*>)-(polyA>)--

EXAMPLE 4

CONSTRUCTION OF A SECOND EXCISION-TYPE AAV PACKAGING CASSETTE COMPRISING A REP52 GENE

As a second illustrative AAV packaging cassette according to the present invention, we constructed an excision-type cassette in which a different AAV packaging gene (*rep52*) is activatably linked to a promoter via an ssr-intervening sequence that comprises a different heterologous gene (*hisD*) (effectively blocking transcription from the promoter). The cassette is constructed such that activation by recombinase results in removal of the ssr-intervening sequence and, in the resulting rearranged cassette, the AAV packaging gene is placed into operable linkage with the promoter, thereby allowing transcription.

This exemplary excision-type AAV packaging cassette was first generated as a plasmid termed "pCMVloxP-loxPRep52". The significant components of pCMVloxP-loxPRep52 are as follows, in order: an pCMV, a first ssr site (loxP site), a second ssr site (loxP site), and an AAV *rep52* gene. The loxP sites are in tandem arrangement. In this embodiment, the *hisD* gene and the adjoining polyA signal represent the ssr-intervening sequence, as they lie between the two loxP sites.

Thus the pCMVloxP-loxPRep52 vector comprises the following components in the relative order and orientation shown below:

--(pCMV>)-(ssr>)-[(*hisD*>)-(polyA>)]-(ssr>)-(*rep52*>)-(polyA>)--

To create the plasmid pCMVloxP-loxPRep52, a 1.6 kb Bcl I/Xho I DNA fragment containing the *rep52* gene including its polyA signal (from the plasmid pMt-rep/cap//pKO-neo as described in a commonly-owned application published as WO 96/17947 on 13 June 1996) was subcloned into the Bgl II/Xho I sites of pCMVloxP-loxP (Example 2). An approximately 2.9 kb NotI-SalI DNA fragment containing the L-histidinol resistance (*HisD*^{r}) gene (including the SV40 polyA signal, as described in Hartmann, S.C., et al., Proc. Natl. Acad. Sci. USA 85: 8047-8051) was subcloned into the Not I/Sal I sites of pCMVloxP-loxPRep52. The resulting construct was denoted pCMVOFFRep52.

The source of the *HisD*^{r} gene was the plasmid pHD-1 (as described in Hartmann et al.,

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id.). A 1.4 kb BamHI DNA fragment containing the *HisD*^{r} gene was subcloned at the BamHI site of pBluescript II SK(-) to create the construct pSK(-)*HisD*^{r}. To add the SV40 polyA signal, the plasmid pREP-8 (Invitrogen) was digested with BspE I and Sal I and a 1.8 kb DNA fragment encompassing the 3' half of the *HisD*^{r} gene followed by an SV40 polyA signal was subcloned into the BspE I/Sal I sites of pSK(-)*HisD*^{r} after removal of a 0.3 kb DNA fragment. The resulting plasmid was denoted as pSK(-)*HisD*^{r} pA. Next, pSK(-)*HisD*^{r} pA was digested with Not I and Sal I, and a 2.8 kb DNA fragment containing the *HisD*^{r} gene (including the SV40 polyA signal) was subcloned into the Not I/Sal I sites of pCMVloxP-loxPRep52, to generate pCMVOFFRep52.

Following activation of the packaging cassette by the corresponding recombinase (i.e. the Cre protein), the ssr-intervening sequence is excised and the rearranged cassette contains the AAV packaging gene in operable linkage with the promoter, as follows:

--(pCMV>)-(ssr)-(*rep52*>)-(polyA>)--

EXAMPLE 5

CONSTRUCTION OF A THIRD EXCISION-TYPE AAV PACKAGING CASSETTE COMPRISING AN AAV *SPLIT-CAP* GENE

As another illustrative embodiment of a AAV packaging cassette according to the present invention, we constructed an excision-type cassette in which a different AAV packaging gene (*split-cap*) is activatably linked to a promoter via an ssr-intervening sequence that comprises a different heterologous gene (*pac*) that effectively blocks transcription from the promoter. The cassette is constructed such that activation by recombinase results in removal of the ssr-intervening sequence and, in the resulting rearranged cassette, the AAV packaging gene is placed into operable linkage with the promoter, thereby allowing transcription.

This exemplary excision-type AAV packaging cassette was generated as a plasmid termed "pCMVOFFCap" using techniques analogous to those described above. The significant components of pCMVOFFCap are as follows, in order: an pCMV, a first ssr site (loxP site), a *pac* gene with a polyA signal (from SV40), a second ssr site (loxP site), and an AAV *split-cap* gene. The ssr sites are in tandem arrangement to promote excision. In this embodiment, the *pac* gene and the adjoining polyA signal represent the ssr-intervening sequence, as they lie between the two loxP sites.

Thus, the pCMVOFFCap vector comprises the following components in the relative order and orientation shown below:

--(pCMV>)-(ssr>)-(*pac*>)-(polyA>)-(ssr>)-(*split-cap*>)-(polyA>)--

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To create the plasmid pCMVOFFCap, a 2.6 kb HindIII/BamHI DNA fragment containing a *split-cap* gene (nucleotides 1881-4496 encoding the Cap protein of AAV, e.g. from plasmid pAV2, Laughlin, C.A., et al., Gene 23:65-73, 1983) followed by an SV40 polyA site was filled in using the Klenow reagent and blunt-end ligated downstream of the loxP sites of pCMVloxP-loxP, which had been previously digested with BglII and XhoI and filled in with Klenow. The resulting plasmid was denoted "pCMVloxP-loxPCAPpA". Next, an approximately 1.2 kb NotI/SalI DNA fragment containing the puromycin resistance gene (*pac*, GENBANK accession number U07648) followed by an SV40 polyA site was cloned into the NotI/SalI sites of plasmid pCMVloxP-loxPCAPpA to generate plasmid pCMVOFFCap.

Following activation of the packaging cassette by the corresponding recombinase (i.e. the Cre protein), the ssr-intervening sequence is excised and the rearranged cassette contains the AAV packaging gene in operable linkage with the promoter, as follows:

--(pCMV>)-(ssr)-(*split-cap*>)-(polyA>)--.

EXAMPLE 6

CONSTRUCTION OF INVERSION-TYPE AAV PACKAGING CASSETTES

As an illustrative embodiment of an inversion-type AAV packaging cassette according to the present invention, techniques as described above can be used to generate an cassette in which an AAV packaging gene (*rep78*) is activatably linked to a promoter via an ssr-intervening sequence that comprises the promoter in an inverted orientation (such that transcription is initially directed away from the AAV packaging gene). The cassette is constructed such that activation by recombinase results in inversion of the ssr-intervening sequence and, in the resulting rearranged cassette, the AAV packaging gene is placed into operable linkage with the promoter, thereby allowing transcription.

This exemplary inversion-type AAV packaging cassette is generated as a plasmid termed "p(invPR)Rep78". The significant components of p(invPR)Rep78 are as follows, in order: a first ssr site (e.g. a loxP site), a promoter (such as the pCMV) orientated such it promotes transcription in an upstream direction (i.e., toward the first ssr site), a second ssr site (e.g. loxP site), and an AAV split-packaging gene (e.g., *rep78*). The ssr sites are in inverted arrangement.

Thus the p(invPR)Rep78 vector contains, in order (arrowheads following the ssr sites represent the orientation of those sites relative to each other):

--(ssr >)-(<promoter)-(ssr <)-(*rep78*>)-(polyA>)--.

Briefly, for construction of an inversion-type AAV packaging cassette, a polynucleotide

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comprising a pair of ssr sites arranged in inverted orientation can be generated essentially using the techniques described above in Example 1 (wherein the second ssr site is inverted relative to the first). Then, following techniques analogous to those in Example 2, a generic vector can be constructed that can be used for the preparation of a variety of such inversion-type cassettes, by cloning a sequence containing a promoter in-between the ssr sites such that the promoter is arranged to promote transcription in the upstream direction (i.e. toward the first ssr site). That generic vector can then be used to generate an inversion-type AAV packaging cassette by cloning an AAV packaging gene into a position downstream of the second ssr site; such that inversion of the ssr-intervening sequence (upon contact with recombinase) results in the promoter being inverted and placed into operable linkage with the downstream AAV packaging gene.

Following activation of p(invPR)Rep78 by the corresponding recombinase, the ssr-intervening sequence is inverted and the rearranged cassette would contain the AAV packaging gene in operable linkage with the promoter, as follows:

--(ssr>)-(promoter>)-(<ssr)-(*rep78*>)-(polyA>)--.

Other AAV packaging genes (such as *rep52*, *split-cap* and/or wild-type AAV packaging genes) can be incorporated in an analogous fashion in place of *rep78*. Similarly, other ssr sites such as FRT sites could be employed in place of loxP sites, as discussed above.

Preferably a terminator is included upstream of the first ssr site to prevent upstream transcription from effecting expression of the *rep78* gene.

EXAMPLE 7

CONSTRUCTION OF A SECOND INVERSION-TYPE AAV PACKAGING CASSETTE COMPRISING AN AAV REP78 GENE

As a second illustrative embodiment of an inversion-type AAV packaging cassette according to the present invention, an cassette is constructed in which an AAV packaging gene (*rep78*) is activatably linked to a promoter by placing the AAV packaging gene within the ssr-intervening sequence in an inverted orientation relative to the promoter (which is outside of the ssr-intervening sequence). The cassette is thus constructed such that activation by recombinase results in inversion of the ssr-intervening sequence and, in the resulting rearranged cassette, the AAV packaging gene is placed into operable linkage with the promoter, thereby allowing transcription.

This exemplary inversion-type AAV packaging cassette is generated as a plasmid termed "pPR(invRep78)". The significant components of pPR(invRep78) are as follows, in order: a

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promoter (such as the pCMV) orientated such that transcription is in the direction of the first ssr site, a first ssr site (e.g. a loxP site), an AAV split-packaging gene (e.g., *rep78*) oriented such that the direction of transcription is toward the first ssr site, and a second ssr site (e.g. loxP site). Preferably the inverted AAV packaging gene is preceded by a transcriptional terminator. The ssr sites are in inverted arrangement.

Thus the pPR(invRep78) vector contains, in order (arrowheads following the ssr sites represent the orientation of those sites relative to each other):

--(promoter>)-(ssr >)-[(<polyA)-(<*rep78*)]-(ssr <)--.

Briefly, for construction of such an inversion-type AAV packaging cassette, a polynucleotide comprising a pair of ssr sites arranged in inverted orientation can be generated essentially using the techniques described above in Example 1. Then, following techniques analogous to those in Example 2, a generic vector can be constructed that can be used for the preparation of a variety of such inversion-type cassettes, by cloning a sequence containing a promoter upstream of the first ssr site (oriented so as to promote transcription toward the ssr sites). That generic vector can then be used to generate an inversion-type AAV packaging cassette by cloning an AAV packaging gene into position as the ssr-intervening sequence but in an orientation such that it faces the upstream promoter (as shown above); whereby activation of the cassette (upon contact with recombinase) results in the AAV packaging gene being inverted and placed into operable linkage with the upstream promoter.

Following activation of pPR(invRep78) by the corresponding recombinase, the ssr-intervening sequence is inverted and the rearranged cassette would contain the AAV packaging gene in operable linkage with the promoter, as follows:

--(promoter>)-(ssr>)-[(*rep78*>)-(polyA>)]-(<ssr)--.

Other AAV packaging genes (such as *rep52*, *split-cap* and/or wild-type AAV packaging genes) can be incorporated in an analogous fashion in place of *rep78*. Similarly, other ssr sites such as FRT sites could be employed in place of loxP sites, as discussed above.

A transcriptional terminator can also be located downstream of the second ssr site in the cassette to block any exogenous transcriptional activity from influencing transcription of the packaging gene in the non-activated state.

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EXAMPLE 8

CONSTRUCTION OF A THIRD INVERSION-TYPE AAV PACKAGING CASSETTE COMPRISING AN AAV REP78 GENE

As a third illustrative embodiment of an inversion-type AAV packaging cassette according to the present invention, an cassette is constructed in which an AAV packaging gene (*rep78*) is activatably linked to a promoter by placing a uni-directional terminator within the ssr-intervening sequence. The cassette is thus constructed such that activation by recombinase results in inversion of the ssr-intervening sequence and, in the resulting rearranged cassette, the AAV packaging gene is placed into operable linkage with the promoter, thereby allowing transcription.

This exemplary inversion-type AAV packaging cassette is generated as a plasmid, the significant components of which are as follows, in order: a promoter (such as the pCMV) orientated such that transcription is in the direction of the first ssr site, a first ssr site (e.g. a loxP site), a uni-directional terminator (such as SV40 polyA) oriented to block transcription originating from the promoter, a second ssr site (e.g. loxP site), an AAV split-packaging gene (e.g., *rep78*) oriented such that the direction of transcription is toward the first ssr site. Preferably the inverted AAV packaging gene is preceded by a transcriptional terminator. The ssr sites are in inverted arrangement.

Thus the vector contains, in order (arrowheads following the ssr sites represent the orientation of those sites relative to each other):

--(promoter>)-(ssr>)-(term>)-(ssr<)-(*rep78*>)-(polyA>)--.

Briefly, for construction of such an inversion-type AAV packaging cassette, a polynucleotide comprising a pair of ssr sites arranged in inverted orientation can be generated essentially using the techniques described above in Example 1 (wherein the second ssr site is inverted relative to the first). Then, following techniques analogous to those in Example 2, a generic vector can be constructed that can be used for the preparation of a variety of such inversion-type cassettes, by cloning a promoter upstream of the first ssr site and a uni-directional terminator in-between the ssr sites such that the terminator is arranged to prevent transcription in the downstream direction (i.e. toward the second ssr site), as shown above. That generic vector can then be used to generate an inversion-type AAV packaging cassette by cloning an AAV packaging gene into a position downstream of the second ssr site; such that inversion of the ssr-intervening sequence (upon contact with recombinase) results in the uni-directional terminator being inverted, thus effectively placing the AAV packaging into operable linkage with the

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upstream promoter. Following activation of the cassette by the corresponding recombinase, the ssr-intervening sequence is inverted and the rearrangcd cassette would contain the AAV packaging gene in operable linkage with the promoter, as follows:

--(promoter>)-(ssr>)-(<term)-(<ssr)-(*rep78*>)-(polyA>)--.

Other AAV packaging genes (such as *rep52*, *split-cap* and/or wild-type AAV packaging genes) can be incorporated in an analogous fashion in place of *rep78*. Similarly, other ssr sites such as FRT sites could be employed in place of loxP sites, as discussed above.

EXAMPLE 9

GENERATION OF PLASMIDS EXPRESSING RECOMBINASE PROTEIN

Enzymatically-active recombinase proteins for use in the present invention can be provided by any of a variety of means as describcd above. Where the recombinase protein is expressed from a gene introduced into the packaging cell, as in various preferred embodiments of the present invention, the gene encoding the recombinase can be carried on any of various replicons in the packaging cell. For example, in a prefcrred embodiment described in more detail below, a recombinase gene is included within the genome of an AAV helper virus that is used to activate AAV replication and encapsidation in the packaging cell. Recombinase genes can alternatively be carried in a cellular chromosome, or on any other replicon within the packaging cell; or the recombinase gene can be introduced into the cell in a manner that results in transient expression of the recombinase in the cell (e.g., by transfection with a plasmid or other vector comprising the recombinase gene), with or without subsequent integration of the recombinase gene into a stably-maintained replicon of the packaging cell.

By way of illustration, in this example, we describe the generation of various plasmids comprising recombinase genes that can be used to express the recombinase directly (e.g., by transient expression in a transfected mammalian cell), or can be used as a source of recombinase genes to be introduced and/or expressed in other ways (e.g., by subcloning the recombinase gene(s) into a helper virus, or directly into a chromosome).

The recombinase activity is preferably initiated in the packaging cell concurrently with the desired initiation of AAV replication/encapsidation functions (e.g. by introducing a recombinase gene as part of the helper virus or by a separate event such as transfection that is closely-associated in time with thc introduction of helper virus, by initiating the expression of a rccombinase gene in concert with the desired initiation of replication/encapsidation, or by initiating the enzymatic activity of a recombinase protein such as by a temperature or other

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environmental shift).

The choice of recombinase gene will necessarily depend on the site-specific recognition sequences selected for incorporation into the AAV packaging cassettes of the present invention. By way of illustration, the Cre recombinase gene was selected to provide a source of protein that can activate cassettes comprising pairs of loxP sites as described above. A number of recombinases are known and their encoding genes have been cloned or are readily obtainable.

We prepared two different versions of genes encoding Cre recombinase; with and without a nuclear localization signal ("NLS") that can be used to enhance the levels of the protein product in the nucleus of a recipient mammalian cell (thereby facilitating interaction between the introduced recombinase and AAV packaging cassettes that may be introduced into the cell nucleus). In particular, without the addition of an NLS, the recombinase protein may not be introduced into the nucleus in substantial amounts except during cell division when the nuclear membrane is absent.

In order to construct these plasmids, a 1031 bp sequence encompassing the ORF of bacteriophage P1 Cre recombinase from the plasmid pHSG-cre (ATCC CRL 87075), was used as a template for PCR. The following oligonucleotides were used as PCR primers: The forward amplimer, 5'-GCGCG GCGGC CGCGG TACCA GATCT ATGTC CAATT TACTG ACCGTA-3' (SEQ ID NO:4), contains a Not I, Asp 718 and Bgl II restriction enzyme sites followed by the first six codons of Cre. The reverse amplimer 5'- GCCGC GCGGC CGCTT ATTAC TAATC GCCAT CTTCC AGCAG GCGCAC-3'

(SEQ ID NO:5), encompasses the last 8 codons of Cre, add two additional UAA termination codons followed by a Not I site. The resulting 1065 bp Cre PCR product was subcloned as a Not I fragment into the Not I site of pCMVβ (after removal of the Not I β-gal gene fragment). The resulting plasmid is designated pCMVCre. To add a nuclear localization signal (NLS) at the N-terminus of Cre, a 79 bp oligo 5'-

GCGCGCGGTACCCAGACCGTGCATCATGAGCGGCCCTCCAAAAAAGAAGA GAAAGGTAGAAGACCCGAGATCTGCGCGC-3' (SEQ ID NO:6) containing an initiation codon fitting Kozak's rule (Kozak, M. 1981. Nucleic Acid Res., 9, 5233-5252) and a coding sequence of NLS from SV40 T antigen, was subcloned between the Asp 718 and Bgl II sites of pCMVCre.

The resulting plasmid is designated pCMVNCre and is introduced by methods known in the art into host cells already carrying AAV packaging cassettes. The recombinase encoded by pCMVNCre mediates recombination of the AAV packaging cassettes, which then express packaging genes necessary to replicate and encapsidate rAAV vectors.

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EXAMPLE 10

GENERATION OF HELPER VIRUS EXPRESSING A RECOMBINASE PROTEIN

In certain preferred embodiments of the present invention, the recombinase protein used to activate the AAV packaging cassettes is expressed from a recombinant AAV helper virus (such as a recombinant adenovirus) that also provides other necessary functions for promoting AAV replication and/or encapsidation.

By way of illustration, recombinant adenovirus comprising recombinase genes (with or without an NLS as described in the preceding example) can be prepared using standard molecular biological techniques. In particular, recombinase genes can be conveniently incorporated into a recombinant adenovirus at a site in the adenoviral genome that is either unnecessary for viral replication and/or helper functions, or at a site encoding a necessary gene product which can be complemented *in trans* (e.g., by providing a copy of the necessary gene in a packaging cell to be used in conjunction with the present invention). By way of illustration, a recombinase gene can be introduced into the non-essential E3 region of adenovirus. Alternatively, a recombinase gene can be introduced into the essential E1 region of adenovirus for use in cells such as human 293 cells which are capable of complementing the E1 deficiency *in trans*. Techniques for facilitating cloning into adenovirus vectors, e.g. into the E1 and/or E3 regions, are known in the art (see, e.g., Bett, A.J., et al., Proc. Natl. Acad. Sci. USA 91:8802-8806, 1994; and Kanegae, Y., et al., Nucl. Acids Res. 23: 3816-3821, 1995, describing generation of the recombinant adenovirus AxCANCre (or AdAxCANCre) in which an NLS-tagged Cre gene is cloned into the E1 region of adenovirus).

Using techniques as described in the art, plasmids such as pCMVCre and/or pCMVNCre can thus be used as sources of recombinase genes to be sub-cloned into another site (such as within the E1 or E3 regions of adenovirus).

We have generated recombinant Ad helper virus in which an NLS-tagged recombinase (Cre) is inserted into the E3 region of adenovirus, which can be used to introduce recombinase protein into any of variety of cells useful as mammalian packaging cells. In particular, since the E1 region of adenovirus is left intact and the E3 region is generally non-essential, it is possible to employ such a recombinant adenovirus to deliver helper virus functions (as well as recombinase) even to cells such as Hela cells which, unlike 293 cells, do not contain adenovirus complementing sequences.

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EXAMPLE 11

INTEGRATING AN AAV PACKAGING CASSETTE COMPRISING AN AAV REP78 GENE INTO A MAMMALIAN CELL

As described above, in preferred embodiments of the present invention, one or more AAV packaging cassettes is stably integrated into a mammalian cell that can be useful as a mammalian packaging cell for the production of rAAV particles. Stable integrants can be readily obtained using standard molecular biological techniques. Inclusion of a selectable marker either within or adjacent to the AAV packaging cassette to be stably introduced can facilitate the selection of cells that obtain and/or retain the AAV packaging cassette.

By way of illustration, we have generated a variety of different mammalian cell lines comprising stably-integrated AAV packaging cassettes according to the present invention. In this example, we describe the integration of an AAV packaging cassette comprising an AAV *rep78* gene into a human cell line (a 293 cell line sometimes referred to as "293-1") into which an rAAV pro-vector had been stably integrated (briefly, this line, referred to as 293AAVCFTR herein, was previously generated by transfecting 293 cells with a plasmid "tgAAVCFTR" which contains the AAV2 inverted terminal repeats (nucleotides 1-145 and 4490-4681) flanking nucleotides 133 to 4573 of the CFTR cDNA (entire coding sequence) and a synthetic polyadenylation signal based on the murine beta-globin at the 3' end, see, Afione, S.A., et al., J. Virol. 70:3235-3241, 1996)) and, in addition, the hygromycin-B-phosphotransferase gene.

Human cells such as 293 cells (ATCC CRL 1573) were generally maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% calf serum and 100 units/ml penicillin and streptomycin. Cells were grown in a humidified 37 degree C incubator supplemented with 10% CO_2. Generally, electroporation was performed using 15-30 micrograms of plasmid DNA for 2-5 x10^6 cells, and was routinely performed in about 800 microliters of serum-free medium in a 0.4 cm cuvette at 210 volts, 960 microF using a Gene Pulser (Bio-Rad). After electroporation, cells were typically plated in a T-75 flask in DMEM comprising a selective component (e.g., 0.65 mg/ml G418 (Gibco-BRL) for cells having a neomycin resistance gene such as a *neo* or *geo* gene). The selected cell population was expanded and maintained in selective medium.

Briefly, human 293AAVCFTR cells (containing integrated tgAAVCFTR) were transfected with pCMVOFFREP78 (as described above). Polyclonal cell lines resistant to G418 were obtained after 10-12 days of drug selection and designated HB C1. Individual clones were picked, expanded into clonal populations and tested for the presence of a stable-integrated AAV

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packaging cassette. A number of stable integrants, typically comprising from one to five integrated copies of the AAV packaging cassette, were readily obtained using such techniques. For these examples, we elected to proceed with clones having only a single full-length copy of the AAV packaging cassette (although clones having multiple integrated copies may well be useful as a means of providing additional sources of the AAV packaging functions).

Further characterization and use of the resulting cell lines is described in more detail below. In addition, we also prepared other mammalian cell lines (including 293, Hela S3 and 293 N3S, as described in more detail below) which comprise stably-integrated AAV packaging cassettes.

EXAMPLE 12

USE OF RECOMBINASE GENES (WITH OR WITHOUT NUCLEAR LOCALIZATION SIGNALS) TO ACTIVATE AN AAV PACKAGING CASSETTE

In this example, we examined the ability of recombinase genes (with or without associated nuclear localization signals) to activate an AAV packaging cassette comprising a *rep78* gene, and to thereby promote the rescue and amplification of an rAAV vector.

Briefly, a population of HB C1 [human 293AAVCFTR cells having a stably-integrated copy of plasmid pCMVOFFRep78 (i.e. a "single integrant" prepared as described above)] (approximately $4x10^6$ cells in a T-75 flask) was transfected by electroporation with 10 micrograms of either pCMVCre or pCMVNCre as described above in Example 9, and then plated in DMEM overnight. The cells were then infected with adenovirus [Ad5 at an moi (multiplicity of infection) of about 5], and incubated for 48 hours at 37 degrees Celsius. Following incubation, we harvested the cells, isolated and purified total DNA, and digested the DNA with EcoR1. Samples were electrophoresed on an agarose gel, and then blotted to a nylon membrane. Blots were subsequently probed by hybridization with a ^{32}P-labeled 1.48 kb EcoRI fragment of the CFTR gene. Positive controls included cells that had been transfected with a plasmid pmMT1Rep (containing the AAV *rep* gene) and then infected with Ad5. Negative controls included cells that did not have an integrated AAV packaging cassette, cells that had not been transfected with a recombinase, and cells that had not been infected with a helper virus.

Figure 1 is a copy of an autoradiograph reflecting hybridization of the CFTR probe to EcoRI-digested DNA isolated from cells carrying constructs of the present invention and control cells as follows: 0 = lambda HindIII marker; 1 = single integrants of CMVOFFRep78 (not infected with Ad and not transfected with any Cre-containing plasmid); 2 = single integrants

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transfected with pCMVCre but not infected with Ad; 3 = single integrants transfected with pCMVCre and infected with Ad; 4 = single integrants transfected with pCMVNCre but not infected with Ad; 5 = single integrants transfected with pCMVCre and infected with Ad; 6 = 293AAVCFTR parental-type cells transfected with pmMT1Rep but not infected with Ad; and 7 = 293AAVCFTR parental-type cells transfected with pmMT1Rep and infected with Ad. The intensely-hybridizing band at about 1.5 kb corresponds to the position expected for the internal EcoRI fragment of CFTR (which is 1.488 kb).

The hybridization results, as shown in Figure 1, revealed that cells into which we had introduced the AAV packaging cassette (and other necessary functions including helper virus) were capable, upon activation by recombinase, of mediating the rescue and amplification of an rAAV pro-vector. As expected, elimination of the AAV packaging cassette, the recombinase or the helper virus infection essentially prevented the effective rescue and amplification of the rAAV pro-vector.

The results also revealed that the introduction of an NLS into the recombinase gene resulted in a substantially enhanced ability to generate rescued amplified rAAV vector. In particular, CFTR-hybridizing DNA bands obtained from cell populations that had been transfected with pCMVNCre were approximately five- to ten-fold more intense than equivalent bands from cells transfected with pCMVCre.

EXAMPLE 13

RESCUE AND AMPLIFICATION OF A RECOMBINANT AAV VECTOR USING A RECOMBINASE-ACTIVATABLE PACKAGING CASSETTE OF THE PRESENT INVENTION

In this example, we examined the ability of a cell line having an AAV packaging cassette comprising a *rep78* gene to promote the rescue and amplification of an rAAV vector.

Briefly, a population of HB C1 cells [human 293AAVCFTR cells having a stably-integrated copy of plasmid pCMVOFFRep78 (prepared as described above)] (approximately $2x10^6$ cells in a T-75 flask) was infected with adenovirus at an moi of about 5 (Ad5, AdAxCANCre or an AdNCre comprising the *cre* gene and an associated Nuclear Localization Sequence (NLS) in the E1 region of Ad, as described in Examples 9 and 10). Following infection, cells were incubated for 1 hour at 37 degrees Celsius, and were then transfected with pCMVCap (15 micrograms). It should be noted that the introduction of the AAV *cap* genes is not required for the rescue and amplification of rAAV vector, but was included so that the same cells

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could be used to test for production of rAAV vector particles (which does require the presence of Cap protein).

After incubation for 48-72 hours, a portion of the cells was harvested and used to obtain DNA. After purification, DNA was digested with EcoR I, blotted as described above, and probed with a CFTR-specific hybridization probe. Samples were electrophoresed on an agarose gel, and then blotted to a nylon membrane. Blots were subsequently probed by hybridization with a ^{32}P-labeled 1.4 kb EcoRI fragment of the CFTR gene. Controls included parental-type cells that were transfected with a plasmid (pRS5 as described in WO 95/13365) or a combination of plasmids (pmMT1Rep, as described in WO 96/17947, and pCMVCap as described above) providing AAV *rep-cap* sequences, and then infected with either Ad5 or AdNCre.

Figure 2 is a copy of an autoradiograph reflecting hybridization of the CFTR probe to EcoRI-digested DNA isolated from cells carrying constructs of the present invention and control cells as follows: 0 = lambda HindIII marker; 1 = double integrants of CMVOFFRep78 and CMVOFFRep52 (as described in Example 15) infected with AdNCre and transfected with pCMVCap; 2 = double integrants of CMVOFFRep78 and CMVOFFRep52 infected with a mixture of AdNCre and Ad5 and transfected with pCMVCap; 3 = double integrants of CMVOFFRep78 and CMVOFFRep52 infected with Ad5 and transfected with a mixture of pCMVNCre and pCMVCap; 4 = single integrants of CMVOFFRep78 infected with AdNCre and transfected with pCMVCap; 5 = single integrants of CMVOFFRep78 infected with a mixture of AdNCre and Ad5 and transfected with pCMVCap; 6 = 293AAVCFTR parental-type cells transfected with pRS5 and infected with AdNCre; 7 = 293AAVCFTR parental-type cells transfected with pRS5 and infected with Ad5; 8 = 293AAVCFTR parental-type cells transfected with a mixture of plasmids pmMT1Rep and pCMVCap and infected with Ad5. The intensely-hybridizing band at about 1.5 kb corresponds to the position expected for the internal EcoRI fragment of CFTR (which is 1.488 kb).

These experiments revealed that cells into which we had introduced the AAV packaging cassettes (and other necessary functions including helper virus) were capable, upon activation by recombinase, of mediating the rescue and amplification of an rAAV pro-vector. Additional confirmation of this finding was obtained with NLS-tagged Cre delivered via AdAxCANCre.

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EXAMPLE 14

GENERATION OF CELL LINES HAVING TWO STABLY-INTEGRATED AAV PACKAGING CASSETTES OF THE PRESENT INVENTION

As discussed above, mammalian packaging cells prepared according to the present invention can and preferably do comprise multiple different AAV packaging cassettes. Preferred cell lines of the present invention comprise at least one AAV packaging cassette comprising an AAV *rep78* gene and comprise a second AAV packaging cassette comprising an AAV *split-cap* gene. Preferred cell lines can also include an AAV packaging cassette comprising an AAV *rep52* gene.

In this example, we illustrate the generation of double integrants comprising a first AAV packaging cassettes having a *rep78* gene and a second AAV packaging cassette having a *rep52* gene. Briefly, double integrants were obtained by expanding a clonal population of cells having a single integrated *rep78* cassette (as in Example 11), and electroporating those cells with a plasmid comprising an AAV packaging cassette having a *rep52* gene (in this example, pCMVOFFRep52, as described in Example 4). Most conveniently, the first and second AAV packaging cassettes will contain different selectable markers to facilitate selection and maintenance of both cassettes. In this case, the *rep78* cassette comprised a β-geo selectable marker and the *rep52* cassette comprised an L-histidinol resistance gene. Individual clones that were capable of growth under both G418 (as indicated above) and L-histidinol (1.2 mM) were then screened for the presence of integrated copies of the corresponding AAV packaging cassettes. As above, cells containing from one to more than five copies of the integrated cassettes are readily obtainable. In this case, however, the single integrant had been first selected for possessing only a single intact copy of the recombinase-activatable *rep78* cassette. Double integrants were likewise screencd to identify cells that comprised a single full-length copy of the recombinase-activatable *rep52* cassette.

Further characterization and use of the resulting cell lines is described in more detail below. In addition, we also prepared double integrants using other mammalian cell lines (including 293, Hela S3 and 293 N3S, as further described below).

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EXAMPLE 15

RESCUE AND AMPLIFICATION OF AAV VECTOR USING CELL LINES HAVING TWO STABLY-INTEGRATED AAV PACKAGING CASSETTES

In this example, we illustrate the use of mammalian cells lines comprising two stably-integrated AAV packaging cassettes to mediate rescue and amplification of an integrated rAAV pro-vector.

Briefly, double integrants (prepared according to Example 14) (approximately $2x10^6$ cells in DMEM medium in a T-75 flask) were infected with an adenovirus (AdNCre or AdAxCANCre at moi of 5 or 10) comprising a recombinase gene (Cre) operably linked to a nuclear localization signal (as described by Kanegae, Y., et al., Nucleic Acids Res. 23:3816-3821, 1995). After infection with adenovirus, the cells were incubated for one hour at 37 degrees Celsius, and then were transfected with pCMVCap (10 to 15 micrograms of plasmid per 2.5×10^6 cells as described above). After 48-72 hours further incubation, a portion of the cells was harvested and used to obtain DNA or used to purify cell lysates. After purification, DNA was digested with EcoR1, blotted as described above, and probed with a CFTR-specific hybridization probe. Controls included parental-type cells transfected with a plasmid (pRS5) or a combination of plasmids (pmMT1Rep and pCMVCap) comprising AAV *rep-cap* sequences (as described in Example 13), and infected with either Ad5 or AdNCre.

The involving double-integrants, as shown in Figure 2 lanes 1-3 (see the description in Example 13,) revealed that cells to which we had introduced the AAV packaging cassettes (and other necessary functions including helper virus) were capable, upon activation by recombinase, of mediating the rescue and amplification of an rAAV pro-vector.

EXAMPLE 16

PRODUCTION OF HEAT-STABLE INFECTIOUS AAV VECTOR PARTICLES USING CELL LINES HAVING STABLY-INTEGRATED AAV PACKAGING CASSETTES OF THE PRESENT INVENTION

In this example, we illustrate the use of mammalian cells lines comprising various stably-integrated AAV packaging cassettes to produce heat-stable infectious rAAV vector particles that can be used transfer a gene of interest to a targeted mammalian cell.

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Single and double integrants (having *rep78* and *rep78* plus *rep52* cassettes, respectively, as described above) were infected with adenovirus and transfected with pCMVCap as described in the preceding example. After transfection, incubation was continued for 48-72 hours. From each T-75 flask, one ml samples of cells in medium were lysed by sonication and the lysate was treated with Benzonase (25 units/ml) and incubated for 30 minutes at 37 degrees Celsius. In a first sample from each flask, the lysate was used without heating in a "Clone-37 Assay" for the detection and quantification of infectious rAAV particles (as described in a commonly-owned application published as PCT Publication WO 96/17947). Briefly, infectious rAAV particles can be used to infect clone 37 cells (which contain an integrated copy of an AAV *rep-cap* cassette in which the *rep* gene is placed under the control of the mMT1 promoter). Since clone 37 cells provide both AAV rep and cap gene functions, an incoming rAAV vector can (in combination with infection by adenovirus) be replicated and thus amplified. Although adenovirus was present in the first sample and had not been inactivated by heating, the use of adenovirus carrying a recombinase gene in the E1 region (which would be proficient in 293 cells which can complement the loss of E1) do not provide sufficient helper functions in other cells such as clone 37 cells (which are derived from Hela cells). Therefore, we routinely included additional adenovirus in the unheated sample as well when performing the clone 37 assay. In a second sample from each flask, the lysate was heated at 56 degrees Celsius for 1 hour, inactivating any adenovirus present in the sample (which thus served as an Ad-minus control). In a third sample, from each flask, the lysate was first heated at 56 degrees Celsius for 1 hour (inactivating adenovirus) and then supplemented with additional adenovirus. Each of the lysates was then used to infect clone 37 cells during a 48-72 hour incubation period. After incubation, total DNA was obtained from the infected cells, purified and digested with EcoRI, electrophoresed on an agarose gel and blotted as described above. Blots were then probed with a CFTR-specific hybridization probe.

Figure 3 is a copy of an autoradiograph reflecting hybridization of the CFTR probe to EcoRI-digested DNA isolated from clone 37 cells that had been infected with viral lysates derived from cells carrying constructs of the present invention and control cells as follows: 0 = lambda marker (digested with HindIII); panel #1 = clone 37 cells infected with lysates from double integrants of CMVOFFRep78 and CMVOFFRep52 (as described in Example 15) that had been infected with AdNCre and transfected with pCMVCap (for each of the numbered panels (1-9), the first lane reflects cells infected with unheated lysate with additional Ad5, the second lane reflects cells infected with heated lysate without additional Ad5, and the third lane reflects cells infected with heated lysate with additional Ad5); panel #2 = clone 37 cells infected with lysates from double integrants of CMVOFFRep78 and CMVOFFRep52 that had been infected with a mixture

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of AdNCre and Ad5 and transfected with pCMVCap; panel #3 = clone 37 cells infected with lysates from double integrants of CMVOFFRep78 and CMVOFFRep52 that had been infected with Ad5 and transfected with a mixture of pCMVNCre and pCMVCap; panel #4 = clone 37 cells infected with lysates from single integrants of CMVOFFRep78 that had been infected with AdNCre and transfected with pCMVCap; panel #5 = clone 37 cells infected with lysates from single integrants of CMVOFFRep78 that had been infected with a mixture of AdNCre and Ad5 and transfected with pCMVCap; panel #6 = clone 37 cells infected with lysates from 293AAVCFTR parental-type cells that had been transfected with pRS5 and infected with AdNCre; panel #7 = clone 37 cells infected with lysates from 293AAVCFTR parental-type cells that had been transfected with pRS5 and infected with Ad5; panel #8 = clone 37 cells infected with lysates from 293AAVCFTR parental-type cells that had been transfected with a mixture of plasmids pmMT1Rep and pCMVCap and infected with Ad5; and panel #9 = clone 37 cells infected with lysates from 293AAVCFTR parental-type cells that had been transfected with a mixture of plasmids pmMT1Rep and pCMVCap and infected with AdNCre. The intensely-hybridizing band at about 1.5 kb corresponds to the position expected for the internal EcoRI fragment of CFTR (which is 1488 base pairs).

The hybridization results shown in Figure 3 revealed that both the single-integrant and double-integrant packaging cell lines could be used to produce heat-stable infectious rAAV vector particles (provided that recombinase and helper virus functions were present), and that the resulting rAAV viral particles could be used to infect target mammalian cells (resulting in substantial amplification of the incoming rAAV vector).

In summary, both single-integrant and double-integrant cell lines comprising AAV packaging cassettes of the present invention could be used to produce heat-stable infectious rAAV viral particles that were capable of mediating transfer of the rAAV vector to mammalian target cells.

EXAMPLE 17

GENERATION OF CELL LINES HAVING THREE STABLY-INTEGRATED AAV PACKAGING CASSETTES OF THE PRESENT INVENTION

As discussed above, preferred cell lines of the present invention comprise at least one AAV packaging cassette comprising an AAV *rep78* gene and comprise a second AAV packaging cassette comprising an AAV *split-cap* gene. Preferred cell lines can also comprise an AAV packaging cassette comprising an AAV *rep52* gene.

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In this example, we illustrate the generation of triple integrants comprising a first AAV packaging cassettes having a *rep78* gene, a second AAV packaging cassette having a *rep52* gene, and a third AAV packaging cassette having a *split-cap* gene. Briefly, triple integrants are obtained by expanding a clonal population of double integrants (as in Example 15), and electroporating those cells with a plasmid comprising an AAV packaging cassette having a *split-cap* gene (in this example, pCMVOFFCap, as described in Example 5). Most conveniently, different AAV packaging cassettes will contain different selectable markers to facilitate selection and maintenance of the cassettes. In this case, the *rep78* cassette comprised a β-geo selectable marker, the *rep52* cassette comprised an L-histidinol resistance gene, and the spit-cap cassette comprised a *pac* gene providing puromycin resistance.

Applying essentially the same procedures as illustrated above, individual clones that are capable of growth under both G418 and L-histidinol (as indicated above), and puromycin (at 0.4 micrograms/ml), are then screened for the presence of integrated copies of the corresponding AAV packaging cassettes. As above, cells containing from one to more than five copies of the integrated cassettes are readily obtainable. In this case, however, the double integrant is first selected for possessing only a single intact copy of the recombinase-activatable *rep78* cassette and a single intact copy of the recombinase-activatable *rep52* cassette (as in Example 14). Triple integrants are likewise screened to identify cells that comprised a single full-length copy of the recombinase-activatable *rep52* cassette.

Such triple integrants need only be infected with a replication-competent helper virus (such as an AdNCre as described above) to generate infectious rAAV vector particles. Triple integrants as described above have been constructed and, as expected, introduction of recombinase into these strains, as described herein, resulted in rescue, replication and packaging of infectious rAAV vector particles.

EXAMPLE 18

GENERATION OF UNIVERSAL PACKAGING CELL LINES COMPRISING AAV PACKAGING CASSETTES

Applying essentially the same techniques as described above, single, double and/or triple integrants can also be readily prepared in other cell lines that can be used for packaging various rAAV vectors. Most conveniently, the present invention can be used to generate "universal" (as opposed to rAAV-specific) cell lines which can then be used for the production of any of a variety of rAAV vectors (i.e. rAAV vectors comprising any of a variety of transgenes of interest).

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By way of illustration, we have used the techniques described above to generate single and double integrants of various AAV packaging cassettes in several different human cell lines. To illustrate using cell lines having several different attributes, we selected human 293 cells which (as noted above), have functions that are capable of complementing adenoviruses carrying heterologous genes in the E1 region; human 293 "N3S" cells, which is a suspension-adaptable cell line derived from 293 cells that would further facilitate the production of rAAV vector using packaging cells in suspension cultures (see, e.g., Graham, F.L., J. Gen. Virol., 68: 937-940, 1987); and Hela S3 cells, which are a suspension-adaptable human Hela cell line (ATCC CCL 2.2). If the adenovirus is to carry the recombinase gene and the packaging cells do not have E1-complementing functions (e.g. with Hela cells), then the packaging cells can be modified by introduction of E1 complementing functions or the recombinase can be introduced into the adenovirus at a different location, preferably in a region such as E3 that is not required for adenovirus replication or helper functions.

Briefly, the techniques described in Example 11 were used to generate single integrants of each of the foregoing cell lines comprising a stably-integrated AAV packaging cassette having a *rep78* gene. Again, the techniques described in Example 15 were then used to generate double integrants of each of the foregoing cell lines comprising two different stably-integrated AAV packaging cassettes (one comprising a *rep78* gene and the other comprising a *rep52* gene).

These single and double integrants can then be used to rescue and amplify rAAV pro-vector using techniques such as those illustrated above with the 293AAVCFTR single and double integrants.

Such universal AAV packaging cell lines prepared according to the present invention can be used to replicate and encapsidate any rAAV vector, provided that the necessary complementing activities (such as helper virus functions and recombinase) are present as described above.

By way of illustration, such universal AAV packaging cell lines can be used to replicate and encapsidate rAAV vector that has been introduced to the cells by any of a variety of methods. For example, rAAV vector can be stably introduced into a universal packaging cell to produce a dedicated rAAV producer cell (as described and illustrated above). Alternatively, the rAAV vector can be introduced to the packaging cell coincident with the initiation of packaging (e.g. introduced on a plasmid or with the helper virus).

Such universal AAV packaging cell lines can also be used to carry out an amplification process referred to as "passage packaging." Briefly, the replication functions that form an integral part of the AAV packaging system can be used to effectively amplify an rAAV vector

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stock since each virus particle that infects a suitable AAV packaging cell can be replicated many fold, and the replicated pro-vectors can then be packaged into a population of progeny rAAV vector particles.

5

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Burstein, Haim

(ii) TITLE OF THE INVENTION: RECOMBINASE-ACTIVATABLE AAV PACKAGING CASSETTES FOR USE IN THE PRODUCTION OF AAV VECTORS

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: PALO ALTO
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94304-1018

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Dylan, Tyler M
(B) REGISTRATION NUMBER: 37,612
(C) REFERENCE/DOCKET NUMBER: 22627-20018.40

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650-813-5600
(B) TELEFAX: 650-494-0792
(C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTAGACCTCC TCAGATTAGC GAGGGGCCAT AGCTTATGAG CTAGCCGC 48

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTAGACCTCC TCAGATTAGC GAGGGGCCAT AGCTTATGAG CTAGCCGC 48

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGAAGGCCTA TAACTTCGTA TAGCATACAT TATACGAAGT TATGCGGCCG CGTCGACATA 60
ACTTCGTATA GCATACATTA TACGAAGTTA TAGATCTCTC GAGAAGCTTA GC 112

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGCGGCGGC CGCGGTACCA GATCTATGTC CAATTTACTG ACCGTA 46

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCGCGGTA CCCAGACCGT GCATCATGAG CGGCCCTCCA AAAAAGAAGA GAAAGGTAGA 60
AGACCCGAGA TCTGCGCGC 79

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CLAIMS

1. A polynucleotide comprising a recombinase-activatable adeno-associated virus (AAV) packaging cassette comprising the following components in the relative order listed from upstream to downstream:

(i) a first site-specific recombination (ssr) site;

(ii) an ssr-intervening sequence; and

(iii) a second site-specific recombination (ssr) site;

wherein the cassette comprises a promoter and an AAV packaging gene selected from the group consisting of an AAV *rep* gene and an AAV *cap* gene,

wherein said promoter is located either within the ssr-intervening sequence or upstream of the first ssr site and said AAV packaging gene is located either downstream of the second ssr site or within the ssr-intervening sequence, and

wherein said promoter is activatably linked to said AAV packaging gene.

2. A polynucleotide comprising an AAV packaging cassette of claim 1, wherein the ssr sites are in tandem orientation relative to each other, the ssr-intervening sequence comprises a terminator, the promoter is located upstream of the first ssr site, and the AAV packaging gene is located downstream of the second ssr site.

3. A polynucleotide comprising an AAV packaging cassette of claim 1 wherein the ssr sites are in inverted orientation relative to each other.

4. A polynucleotide comprising an AAV packaging cassette of claim 3, wherein the promoter is located within the ssr-intervening sequence, the AAV packaging gene is located downstream of the second ssr site, and the promoter and the AAV packaging gene are in divergent orientation relative to each other.

5. A polynucleotide comprising an AAV packaging cassette of claim 3, wherein the AAV packaging gene is located within the ssr-intervening sequence, the promoter is located upstream of the first ssr site, and the promoter and the AAV packaging gene are in convergent orientation relative to each other.

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6. A polynucleotide comprising an AAV packaging cassette of claim 3, wherein the ssr-intervening sequence comprises a uni-directional transcriptional terminator blocking transcription from the promoter in the direction of the first ssr site to the second ssr site, the promoter and the AAV packaging gene are in tandem orientation relative to each other, the promoter is located upstream of the first ssr site, and the AAV packaging gene is located downstream of the second ssr site.

7. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the promoter is a heterologous promoter.

8. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 7, wherein the promoter is a constitutive promoter.

9. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 7, wherein the promoter is an inducible promoter.

10. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 7, wherein the promoter is selected from the group consisting of a CMV promoter, an SV40 promoter and an mMT-1 promoter.

11. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the first and second ssr sites are selected from ssr sites recognized by a recombinase protein selected from the group consisting of a viral recombinase, a prokaryotic recombinase and a eukaryotic recombinase.

12. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 11, wherein the first and second ssr sites are selected from ssr sites recognized by a viral recombinase.

13. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 12, wherein the viral recombinase is a bacteriophage recombinase.

14. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 11, wherein the recombinase is a Cre recombinase of bacteriophage P1.

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15. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 11, wherein the recombinase is a FLP recombinase of *Saccharomyces cerevisiae*.

16. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the ssr-intervening sequence comprises a transcriptional terminator.

17. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the ssr-intervening sequence comprises a marker gene selected from the group consisting of a detectable marker gene and a selectable marker gene.

18. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 17, wherein the terminator sequence comprises a selectable marker gene encoding a selectable marker selected from the group consisting of a positive selectable marker, a negative selectable marker and a bifunctional positive-negative selectable marker.

19. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the AAV packaging gene comprises an AAV *rep* gene.

20. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 19, wherein the AAV packaging gene is selected from the group consisting of an AAV *rep* gene coupled to an AAV *cap* gene, an AAV *rep78* gene, and an AAV *rep52* gene.

21. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the AAV packaging gene comprises an AAV *cap* gene.

22. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 21, wherein the AAV packaging gene is selected from the group consisting of an AAV *cap* gene coupled to an AAV *rep* gene and an AAV split-*cap* gene.

23. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the AAV packaging gene is an AAV split-packaging gene.

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24. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, wherein the AAV packaging gene is an AAV *rep* gene coupled to an AAV *cap* gene.

25. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 23, wherein the AAV packaging gene is an AAV *rep78* gene.

26. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 23, wherein the AAV packaging gene is an AAV *rep78* gene and the promoter is a heterologous promoter.

27. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 23, wherein the AAV packaging gene is an AAV *rep52* gene.

28. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 23, wherein the AAV packaging gene is an AAV split-*cap* gene.

29. A polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, further comprising an enhancer, wherein the enhancer is activatably linked to the AAV packaging gene.

30. A method for generating a polynucleotide comprising an AAV packaging gene operably linked to a promoter, the method comprising contacting a polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1, with a recombinase protein specific for the first and second ssr sites of the recombinase-activatable AAV packaging cassette.

31. A helper virus for AAV having a polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1.

32. A mammalian cell having a polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1.

33. A mammalian cell according to claim 32, wherein the recombinase-activatable AAV

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packaging cassette is stably integrated into the cell.

34. A mammalian cell according to claim 32, the cell comprising at least two different recombinase-activatable AAV packaging cassettes, each of which comprises a different AAV packaging gene.

35. A mammalian cell useful for high efficiency packaging of an rAAV vector according to claim 32, the cell comprising an AAV helper virus.

36. A mammalian cell according to claim 32, the cell comprising an rAAV vector.

37. A mammalian cell according to claim 36, the rAAV vector comprising AAV inverted terminal repeats flanking a heterologous non-AAV gene of interest that is operably linked to a promoter.

38. A mammalian cell according to claim 37, wherein the heterologous gene of interest is a therapeutic gene.

39. A mammalian cell according to claim 38, wherein the therapeutic gene encodes a cystic fibrosis transmembrane regulator.

40. A mammalian cell according to claim 32, wherein the mammalian cell is capable of producing at least about 100 recombinant AAV particles per cell.

41. A mammalian cell according to claim 32, wherein the mammalian cell is capable of producing at least about 200 recombinant AAV particles per cell.

42. A mammalian cell according to claim 32, wherein the cell is capable of producing at least about 400 recombinant AAV particles per cell.

43. A mammalian cell according to claim 32, wherein the mammalian cell is a human cell.

44. A method of generating a mammalian cell useful for packaging of an AAV vector,

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comprising introducing into the cell a polynucleotide comprising a recombinase-activatable AAV packaging cassette according to claim 1.

45. A mammalian cell line useful for high efficiency packaging of an rAAV vector, generated according to the method of claim 44.

46. A method of producing a recombinant AAV particle, comprising incubating a mammalian cell according to claim 32, wherein the mammalian cell comprises a recombinant AAV vector and a gene encoding a recombinase specific for the first and second ssr sites of the recombinase-activatable AAV packaging cassette.

47. A method of producing a recombinant AAV particle according to claim 46, further comprising introducing an AAV helper virus to the mammalian cell.

48. A method of producing a recombinant AAV particle according to claim 47, wherein the AAV helper virus is an adenovirus.

49. A method of producing a recombinant AAV particle according to claim 47, wherein the helper virus comprises a gene encoding a recombinase specific for the first and second ssr sites of the recombinase-activatable AAV packaging cassette.

50. A recombinant AAV particle, produced according to the method of claim 46.

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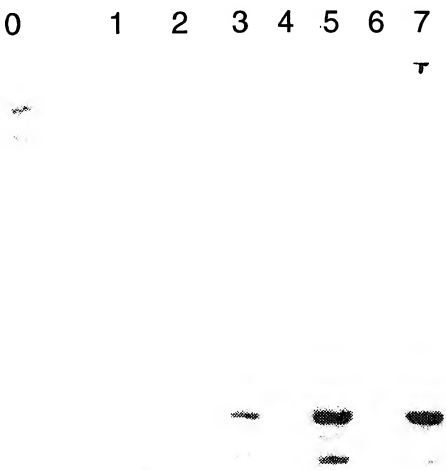

Figure 1.

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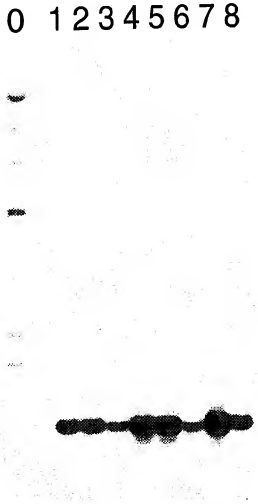

Figure 2.

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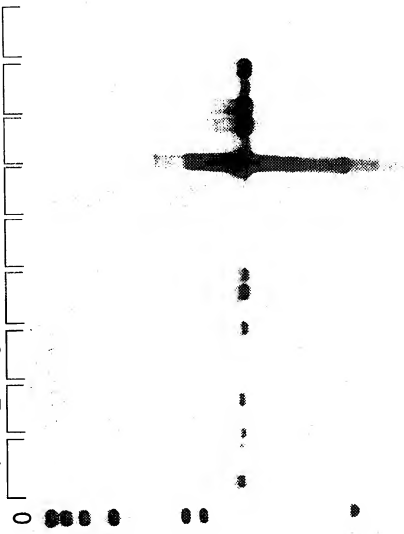

Figure 3.

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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 97/23018

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/85 C12N15/86 C12N9/00 C12N7/01
C12N5/10 C07K14/015 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 98 10086 A (UNIV PENNSYLVANIA ;PHANEUF DANIEL (US); WILSON JAMES M (US)) 12 March 1998 see the whole document	1,2, 7-14, 16-21, 24-27, 29-32, 35-38, 40-50
A	KANEGAE Y ET AL: "EFFICIENT GENE ACTIVATION IN MAMMALIAN CELLS BY USING RECOMBINANT ADENOVIRUS EXPRESSING SITE-SPECIFIC CRE RECOMBINASE" NUCLEIC ACIDS RESEARCH, vol. 23, no. 19, 11 October 1995, pages 3816-3821, XP002011774 see the whole document	1-50

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 May 1998	25/05/1998

Name and mailing address of the ISA
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INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 97/23018

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	ANTON M ET AL: "SITE-SPECIFIC RECOMBINATION MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING THE CRE RECOMBINASE PROTEIN: A MOLECULAR SWITCH FOR CONTROL OF GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 69, no. 8, August 1995, pages 4600-4606, XP002011775 see the whole document ---	1-50
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A	WO 96 17947 A (TARGETED GENETICS CORP ;ALLEN JAMES M (US)) 13 June 1996 see the whole document ---	1-50
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P,A	WO 97 06272 A (AVIGEN INC) 20 February 1997 see the whole document -----	1-50

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/US 97/23018

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